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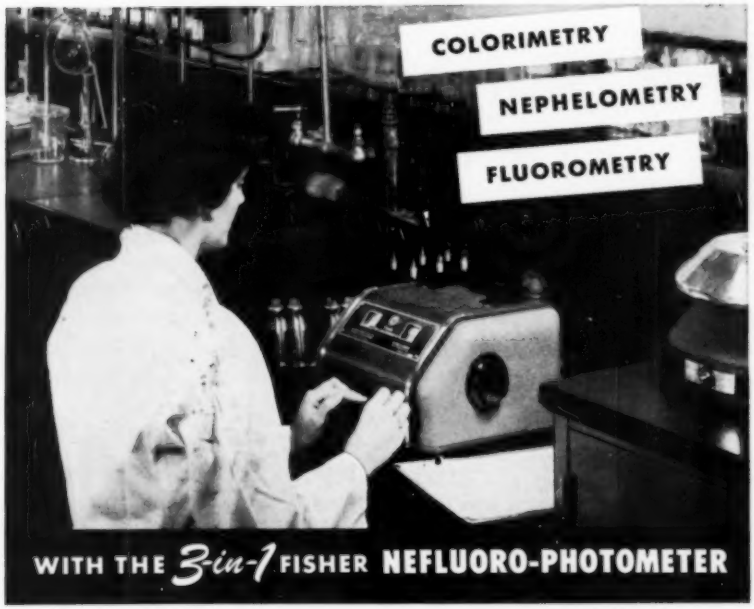

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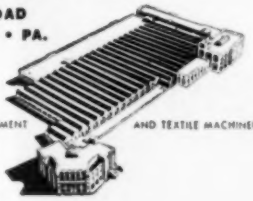

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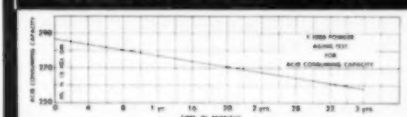

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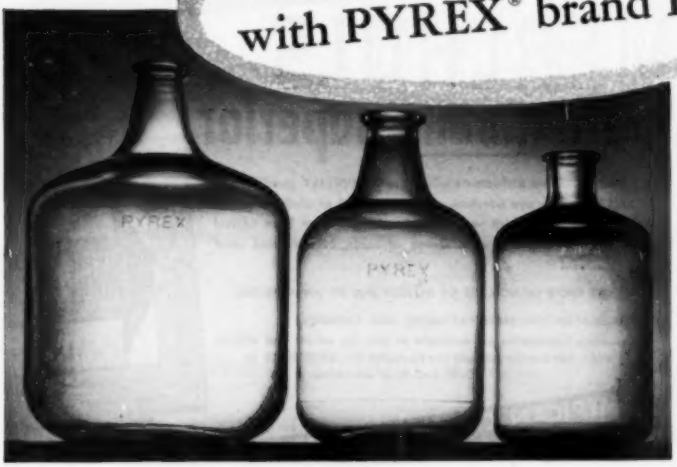

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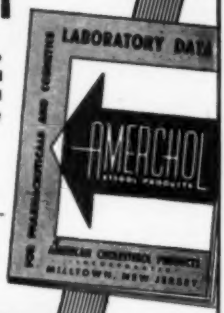

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VOLUME XLI — *SEPTEMBER, 1952* — NUMBER 9 — CONSECUTIVE NO. 18

The Use of Ion-Exchange Resins with Glycosides*,†

By M. A. CHAMBERS,‡ L. P. ZILL, and G. R. NOGGLE§

The comparative behavior of several glycosides on strong-base anion-exchange resins in the chloride and borate forms has been studied. Attempts to extend the borate method of Khym and Zill for the separation of sugars to the separation of glycosides were successful due to the adsorption of the aglycon moiety rather than to exchange adsorption of borate complexes of the glycosides.

EXISTING METHODS of glycoside separation (1–3), in general, lack simplicity as well as definition. The methods are based largely upon the differential solubility of the glycoside and are usually followed by precipitation or crystallization as a purification step. These procedures are not without additional difficulties, since sugars and other substances are frequently obtained with the glycoside. In certain cases, it may become necessary to ferment the sugars or to convert them to some derivative which can be precipitated. Since glycosides display such variable chemical and physical properties, no general method of separation having wide applicability has been developed.

Adsorption techniques have been employed in the preparation of some glycosides. A new glycoside, digicorin, has been separated from digitalis by adsorption on an alumina column (4). More recently, cation-exchange resins have been used for the preparation and purification of flavonoid compounds and their glycosides. In this manner, quercetin has been separated from commercial rutin (5), and morin has been isolated in the pure form from a plant extract (6).

Recently a new method of sugar separation utilizing an ion-exchange technique has been reported (7). It has long been known that boric acid or its salts react with certain polyhydroxy compounds to form anionic complexes (8). Taking advantage of this reaction, Khym and Zill (9) have devised a method whereby sugars can be separated by elution of their borate complexes from strong-base anion exchangers with borate solutions, boric acid-borate buffers, or more recently by chloride and other ions (Khym, J. X., personal communication).

Since glycosides represent a combination of a sugar, or sugars, with an aglycon or nonsugar moiety, it appeared that the method might be applicable to their separation. Inspection of the structural formulas of several glycosides showed that if a borate complex were formed, it would be the weakly ionized type such as that formed by sucrose (10). Experimental evidence to be presented here shows that the behavior of glycosides containing an aliphatic aglycon unit could be explained on this basis but that the

* Received June 2, 1952, from the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

† Work performed under contract No. W-7405-ENG-26 for the Atomic Energy Commission.

‡ Research Participant. Present address: Southern College of Pharmacy, 223 Walton Street, Atlanta, Ga.

§ The authors are indebted to Dr. D. G. Doherty for the synthesis of the methyl-β-D-arabinopyranoside and benzyl-β-D-arabinopyranoside and for helpful discussions during the course of this work.

adsorption of the glycosides containing an aromatic aglycon was too great to be explained in this manner. Although a slight amount of exchange adsorption may take place with such glycosides, it appears likely that the process is almost entirely due to a nonexchange type of adsorption.

EXPERIMENTAL

The apparatus and procedure employed are essentially the same as those used by Khym and Zill (9) except where otherwise noted. The column used is described by Tompkins (11) and by Tompkins, *et al.* (12). The effluent fractions were collected with an automatic fraction collector and the substances were assayed as specified.

The strong-base anion exchanger used was Dowex-1 (200–400 mesh), which was obtained in the chloride form. This resin was used without treatment for the experiments requiring the chloride form of the resin. For experiments requiring the borate form, the resin was washed with distilled water and converted to the borate form by passing 0.1 *M* potassium tetraborate through it until the effluent gave only a slight test for chloride ion. The excess borate was then removed by washing with distilled water.

In order to maintain comparable alkalinity to the borate runs (*p*H 9), the samples which were placed on chloride columns were prepared in 0.01 *N* ammonium hydroxide (*p*H ca. 9).

Arbutin, Salicin, and Saponin.—Arbutin (m. p. 197–199° uncorr.), salicin (m. p. 199–200° uncorr.), and a saponin (*Quillaja saponaria*) were chromatographed on columns in the chloride and borate forms as given in Fig. 3. Arbutin was assayed by measurement of the absorption at 285 mμ, and the salicin and saponin were assayed by the anthrone method (13, 14).

Digitoxin (U. S. P.).—The insolubility of digitoxin in aqueous solution presented a problem which was overcome by dissolving the substance in equal volumes of water and 95% alcohol. The column was previously washed with a solution of the same alcoholic concentration and then with 0.001 *M* potassium tetraborate solution of the same alcoholic concentration. Twenty-five milliliters of an alcohol solution containing 14.9 mg. of digitoxin was mixed with 25 ml. of 0.001 *M* potassium tetraborate solution and placed on the column. This was eluted with 0.015 *M* potassium tetraborate in a 50% v/v water-alcohol solution. The digitoxin was determined qualitatively by the orcinol method (15).

Gitoxin.—This substance was treated in the same manner as the digitoxin; however, in this case, 10 ml. of a saturated solution (50% v/v water-alcohol) containing ca. 5 mg. of gitoxin was mixed with 10 ml. of 0.001 *M* potassium tetraborate, and elution was carried out with 0.001 *M* potassium tetraborate in a 50% v/v water-alcohol solution and determined qualitatively by the orcinol method.

Methyl-β-D-arabinopyranoside and Benzyl-β-D-arabinopyranoside.—These two synthetic glycosides were chromatographed as given in Fig. 2. Assay was made by the orcinol method.

RESULTS

When the anthrone test was applied to salicin and a saponin (*Quillaja saponaria*) in the concentration range of 0–80 μg./ml., a linear relationship was found between concentration and depth of color produced. This conformity with Beer's law thus permits the use of this method for the quantitative determination of the two glycosides.

A study of the absorption characteristics of arbutin solutions demonstrated an absorption peak in the ultraviolet region with a maximum at 285 mμ. The absorption curve is given in Fig. 1. The absorption at 285 mμ was found to vary directly with concentration (in the range of 0–200 μg./ml.), and therefore it may be used for the quantitative determination of arbutin.

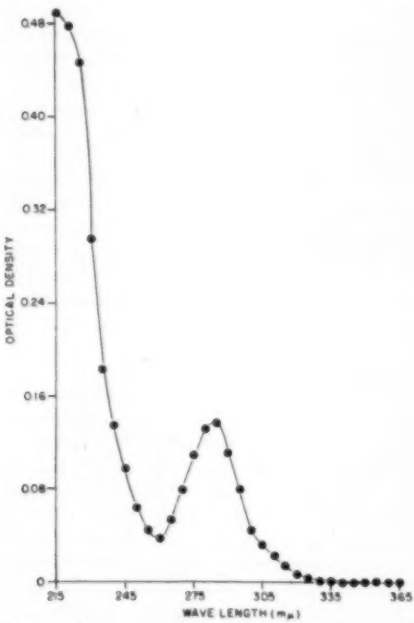

Fig. 1.—Absorption curve for arbutin (20.2 μg./ml.).

When the orcinol test was applied to solutions of digitoxin and gitoxin, a green color appeared which faded with time. This test was used only as a qualitative assay for the detection of the two glycosides. Since neither of these substances was found to be retained by an ion-exchange column, no attempt was made to develop the method quantitatively.

The elution curves for the removal of methyl-β-D-arabinopyranoside and benzyl-β-D-arabinopyranoside from columns in the chloride and borate forms are given in Fig. 2.

The elution of certain glycosides from strong-base anion-exchange resins in the borate and chloride forms is shown by the elution curves for arbutin, salicin, and a saponin (*Quillaja saponaria*) as presented in Fig. 3. The volume-to-peak values (i. e., the volume of eluting agent passed

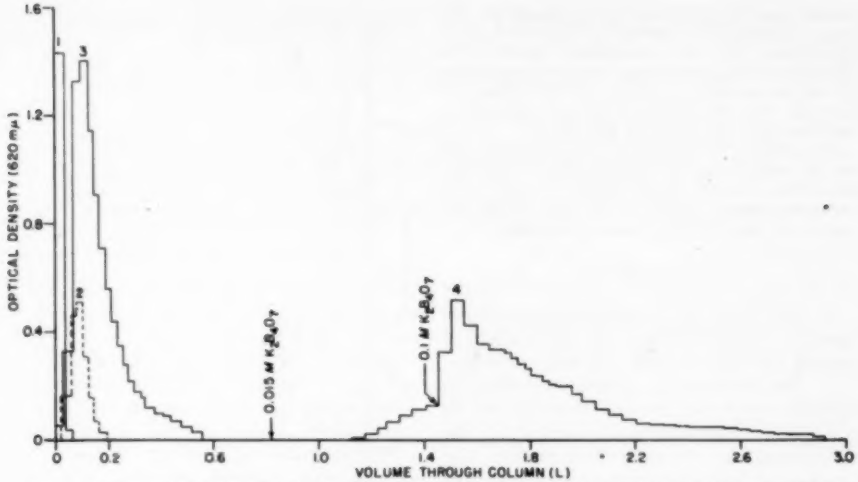

Fig. 2.—Comparative behavior of methyl-β-D-arabinopyranoside and benzyl-β-D-arabinopyranoside on strong-base anion-exchange resins in the chloride and borate forms. Exchanger: Dowex-1, 0.85 sq. cm. x 11 cm., 200–400 mesh, in chloride form for curves 1 and 3 (0.01 *N* ammonium chloride in 0.01 *N* ammonium hydroxide eluting agent); column in borate form for curves 2 and 4 (0.005 *M* potassium tetraborate followed by indicated concentrations as eluting agent). Curves 1 and 2: 10 mg. each of methyl-β-D-arabinopyranoside. Curves 3 and 4: 10 mg. each of benzyl-β-D-arabinopyranoside. Curves 1 and 2 drawn to 0.1 scale. Flow rate: 0.7 to 0.9 ml./min.

through the column to reach the middle of the elution peak) are 2,200 ml., 335 ml., and 240 ml. respectively for the borate column, and 90 ml., 62 ml., and less than 38 ml. (breakthrough) respectively for the chloride column.

Digitoxin and gitoxin, when placed on an anion-exchange column in the presence of borate ions, were not adsorbed, the entire sample appearing in the first one or two fractions. No further orcinol test was obtained with an eluted fraction even after regeneration of the column with 0.1 *M* potassium tetraborate, so that it must be assumed that neither direct adsorption nor exchange adsorption by complex formation occurs with these two glycosides.

DISCUSSION

In order to discuss more adequately the behavior of the natural glycosides on anion-exchange columns, it is well to consider first the behavior of the two synthetic glycosides which were studied. In the case of the methyl-β-D-arabinopyranoside, it was found that this glycoside was easily eluted (i. e., a low volume-to-peak value) with dilute borate solution from a column in the borate form. This behavior is similar to that of sucrose, which forms a weakly ionized borate complex. However, in contrast to sucrose, which is unable to form a strong borate complex because of a lack of *cis*-hydroxyl groups, this compound contains a pair of *cis*-hydroxyl groups (on carbons 3 and 4) which are incapable of forming a highly dissociated complex. Such behavior has been attributed by Böeseken (8) to the repulsion of the *cis*-hydroxyl groups in a pyranose ring so that they assume positions unfavorable to the formation of a borate complex. Replacement of the aliphatic aglycon (methyl group) with a benzyl group giving benzyl-β-D-arabinopyranoside results in a decided increase in affinity for the strong-base anion exchanger in the borate form. An explanation for this increase in affinity based on the exchange adsorption of a borate complex of the glycoside is not possible, since the borate complex must necessarily be identical with the one formed by the aliphatic glycoside. It would therefore appear that the introduction of an aromatic aglycon moiety leads to the direct adsorption of the glycoside by the highly aromatic resin. Such an adsorption on a cation resin has already been mentioned in the introductory remarks.

When the two synthetic glycosides were placed on a strong-base anion exchanger in the chloride form, it was found that the methyl glycoside was not adsorbed at all while the aromatic glycoside was adsorbed only slightly. Thus it seems probable that the increased adsorbent power of the resin in the borate form can be attributed either to the presence of the borate ion itself, or to the absence of the highly polar chloride ion.

On the basis of the previously discussed experiments, a partial interpretation of the behavior of the three naturally occurring glycosides may be given. Inspection of the structural configurations of arbutin, salicin, and saponin shows that, in all three cases, only a weakly ionized borate complex may be produced in the presence of borate ions. Therefore, it must be tentatively concluded that their affinity for the resin is almost entirely due to their non-exchange adsorption. This interpretation does not, however, detract from the use of this adsorption as an aid in the analysis and separation of such glycosides. The application of the method to a

specific problem has been demonstrated by the assay of arbutin in extracts of bearberry plant (Chambers, M. A., unpublished data). The observation that a strong-base anion-exchange resin in the borate form exhibited greater adsorptive capacity than the chloride form was repeated for the three naturally occurring glycosides.

The nonadsorption of digitoxin and gitoxin cannot be explained at the present time. This nonadsorption may be useful, however, since contaminating sugars would be removed by their adsorption on the column as borate complexes.

SUMMARY

1. The use of the anthrone method for the quantitative determination of salicin and a saponin (*Quillaja saponaria*) is demonstrated.

2. The behavior of methyl-β-D-arabinopyranoside and benzyl-β-D-arabinopyranoside on strong-base anion-exchange columns in the chloride and borate forms has been studied. The formation of borate complexes of such glycosides has been shown to be slight; the affinity of glycosides containing aromatic aglycon units is presumably due largely to nonexchange adsorption and only slightly to exchange adsorption. The borate ion has been shown to enhance the adsorptive properties of a strong-base anion-exchange resin for the aromatic glycosides studied.

3. Experiments have been described which show that the glycosides, arbutin, salicin, and a saponin may be adsorbed on a strong-base anion-exchange resin in the borate form and subsequently eluted with potassium tetraborate solution. Adsorption of these three glycosides on a strong-base anion-exchange resin in the chloride form under the conditions employed is not significant for separative purposes.

4. Digitoxin and gitoxin are not adsorbed on a strong-base anion exchanger in the presence of borate ions.

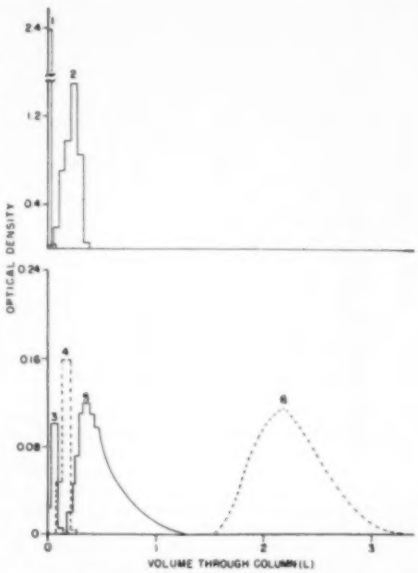

Fig. 3.—Comparative behavior of arbutin, salicin, and a saponin (*Quillaja saponaria*) on a strong-base anion-exchange resin in the chloride and borate forms. Exchanger: Dowex-1, 0.85 sq. cm. x 11 cm., 200–400 mesh, in chloride form for curves 1, 3, and 4 (0.01 *N* ammonium hydroxide in 0.01 *N* ammonium chloride eluting agent) and in borate form for curves 2, 5, and 6 (0.015 *M* potassium tetraborate eluting agent). Curve 1: 5.8 mg. saponin. Curve 2: 20 mg. saponin. Curve 3: 3.6 mg. salicin. Curve 4 (drawn to 0.1 scale): 10 mg. arbutin. Curve 5 (drawn to 0.1 scale): 30 mg. salicin. Curve 6: 15.2 mg. arbutin. Flow rate for all runs was 0.7 to 0.9 ml./min.

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Solvent Considerations in Fungicidal Testing*

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In vitro methods of fungicidal testing involving dermatophytic organisms have varied widely, and the accumulation of comparative data is sometimes difficult. The solvent plays an important part in any test procedure, and a screening of several common solvents indicates the relative nontoxicity of the glycols, in particular, polyethylene glycol 400. Comparative fungicidal data is presented on a number of phenolic chemicals using 95 per cent ethanol and 60 per cent polyethylene glycol 400 as solvents and the mycelial disk technique of fungicidal evaluation.

THE AMOUNT of work performed in scientific circles on the activities of the dermatophytes has been prodigious. *In vitro* methods of testing have varied widely, and it is difficult to compare the effectiveness of a wide variety of fungicides or suspected fungicides where markedly different techniques are used by various investigators. Such appears to be the case in regard to the ringworm infections. This alarming situation led the Council on Pharmacy and Chemistry of the American Medical Association in 1945 to issue a warning statement regarding the advisability of establishing a standard test method for the *in vitro* testing of antifungal compounds (1).

The solvent plays an important part in any *in vitro* testing procedure (2–5), and it is not always possible to use water because of the limited solubility factor. Many substances have been used, such as alcohol, acetone, and dioxane. Besides their inherent lethality, varying results are obtained with different solvents. The desirability of securing a standard substance with adequate solvent power and low toxicity toward the test organism led to the investigation of the possibilities of polyethylene glycol 400. In addition to desirable solvent properties in the testing phase, this glycol is widely used in washable-type ointment bases where again its solvent action may be of advantage in dissolving a given fungicide or fungistat.

EXPERIMENTAL

Conidial Tests.—The present work was particularly concerned with the mycelial-disk testing technique, using polyethylene glycol 400 as solvent; but it was interesting to observe the effect of certain glycols and glycol-phenol concentrations on the conidia of *Trichophyton mentagrophytes*.[1] A glycol solvent concentration of 60% was selected, since solution tests on a large number of chemicals using polyethylene glycol 400 and propylene glycol showed this concentration to be effective without undue viscosity. The test method was that of the U. S. Department of Public Health (6), and the results are shown in Table I. The tables in this paper will contain the annotation "+" which indicates growth of the organism, and "−" which indicates no growth.

TABLE I.—EFFECT OF VARYING CONCENTRATIONS OF PHENOL ON CONIDIAL SUSPENSIONS OF TRICHOPHYTON MENTAGROPHYTES AT AN EXPOSURE OF T MINUTES

	Per Cent (w/v) of Phenol				
Solvent	1	1.25	1.66	2.22	3
Distilled water	+	+	+	−	−
60% Ethylene glycol	+	+	+	+	+
60% Propylene glycol	+	+	+	+	+
60% Polyethylene glycol	+	+	+	+	+

The results with all three glycols were similar and at variance with those obtained using water as the solvent. Another series of tests was run to ascertain the susceptibility of the conidia to polyethylene glycol 400 dilutions and to polyethylene glycol-phenol combinations in order to see if the glycol concentration had a bearing on the results obtained in Table I (see Tables II and III).

TABLE II.—EFFECT OF VARYING CONCENTRATIONS OF POLYETHYLENE GLYCOL 400 ON THE CONIDIA OF TRICHOPHYTON MENTAGROPHYTES

	Exposure, Min.			
Glycol, %	15	30	45	60
25	+	+	+	+
50	+	+	+	+
75	+	+	+	+
100	+	+	+	−

TABLE III.—EFFECT OF 2% (W/V) PHENOL IN VARYING CONCENTRATIONS OF POLYETHYLENE GLYCOL 400 ON CONIDIAL SUSPENSIONS OF TRICHOPHYTON MENTAGROPHYTES

	Exposure, Min.			
Solvent	10	15	20	30
20% Glycol	+	+	+	+
60% Glycol	+	+	+	+
100% Glycol	+	+	+	+

* Received August 31, 1951, from the School of Pharmacy, Purdue University, West Lafayette, Ind.
Presented to the Scientific Section, A. PH. A., Buffalo meeting, August, 1951.
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[1] The authors wish to thank the Ontario Department of Public Health for supplying the organism used in this investigation.

TABLE IV.—EFFECT OF VARIOUS GLYCOL CONCENTRATIONS ON THE ISOLATED MYCELIAL DISK OF TRICHOPHYTON MENTAGROPHYTES AND MICROSPORUM AUDOUINI

Organism	Ethylene Glycol		Propylene Glycol		Polyethylene Glycol 400	
	%	Min.[a]	%	Min.[a]	%	Min.[a]
T. mentagrophytes	25	60	25	60	25	60
	50	60	50	60	50	60
	75	60	75	45	75	60
	100	30	100	30	100	60
M. audouini	25	60	25	60	25	60
	50	60	50	60	50	60
	75	45	75	30	75	60
	100	45	100	15	100	60

[a] Maximum time at which the organism survived for that specific concentration.

It appears from the above tables that the glycols markedly influence the results obtained when using phenol as the test chemical. Many investigators frequently use propylene glycol for its solvent properties in performing conidial and other fungous tests, and it is possible that chemicals other than phenol might appear less efficacious when tested in this manner.

Mycelial Disk Technique.—The use of mycelial sections (5, 7, 8) has many advantages as a strictly *in vitro* testing procedure, principally that it uses the fungus in its actual state of growth. In addition, the resistance of a mycelial mat of *Trichophyton mentagrophytes* to aqueous phenol is decidedly greater than in the case of conidia. Growth is still manifest after an exposure of thirty minutes to a 3% solution of phenol using the test method of Golden and Oster (5).

The following solvents were evaluated for fungicidal activity against *Trichophyton mentagrophytes* and *Microsporum audouini* using the Golden and Oster isolated disk technique with a modification in the rinsing phase: ethylene glycol, propylene glycol, and polyethylene glycol 400 in 25, 50, 75, and 100% (v/v) solutions with distilled water. The times of exposure at each concentration were fifteen, thirty, forty-five, and sixty minutes. No attempt was made to secure an exact lethal concentration for a given time of exposure, but rather an estimate as to relative fungicidal activity. Following treatment, the disks were rinsed for fifteen minutes in sterile distilled water and planted (mycelial surface down) on slants of sterile Sabouraud's agar. Observations for growth were final after twenty-one days (Table IV).

The above results clearly indicate the almost complete lack of toxicity manifested by the glycols using this particular technique. The tests were conducted under somewhat trying conditions, in that such long exposures tend to wash away some of the mycelium, although the agar itself remains intact. The volatile solvents such as ethanol, acetone, or dioxane are much easier to work with under these conditions since they appear to fix the mycelium and prevent washing away.

Fungicidal Tests.—The solvent of choice for fungicidal testing would appear to be one that shows no toxicity to the organism and which is an efficient solvent for a wide range of chemicals. It should also be water-miscible and thus render the process of rinsing much easier in the disk technique. Such properties are manifested by the glycols, particularly polyethylene glycol 400. In order to ascertain the value of this glycol as a solvent in isolated disk test procedures, it was decided to test a series of chemicals, the majority of them phenolic in structure,[1] in a 60% glycol solution and to use 95% ethanol as a comparative solvent. The organism was *Trichophyton mentagrophytes*. The chemicals were tested at a concentration of 1% in both solvents, and the immersion time of the disk was two minutes and five minutes in the glycol and two minutes in the 95% ethanol. Following treatment, the disks were rinsed for five minutes in polyethylene glycol 60%, then for five minutes in 30% acetone-water, and finally for three minutes in sterile broth. Subcultures were made on slants of Sabouraud's dextrose agar and observed for growth after twenty-one days (Table V).

The nuclear halogenated phenols gave comparable results in both solvents, but with other compounds which showed fungicidal activity in alcohol at two minutes, a five-minute exposure in the glycol failed to show fungicidal activity except in three cases. Those chemicals showing no fungicidal activity in the glycol after five minutes were tested again under similar conditions but with exposures of ten and fifteen minutes. Salicylanilide and oxyquinoline sulfate were the only compounds effective after ten minutes, while at fifteen minutes, 2-Br-4-phenylphenol proved fungicidal.

DISCUSSION

From the limited number of tests performed, the use of polyethylene glycol as a solvent in fungicidal testing would appear questionable. It apparently reduces the fungicidal action of phenol by the conidial method. The glycol appears slow to penetrate the mycelial mat and agar disk, which could be attributed to its viscosity and lack of wetting ability. This is in distinct contrast to the volatile materials, such as alcohol, which penetrate very rapidly and thus give higher fungicidal values. *o*-Aminophenol was not effective as a fungicide, using the disk technique and polyethylene glycol as solvent, even after thirty minutes, while in 95 per cent ethanol it was active at 1 per cent after one minute. This apparent discrepancy is readily

[1] The authors wish to thank Dr. G. F. Wright, Department of Organic Chemistry, University of Toronto, for supplying many of the chemicals used in this and other investigations.

TABLE V.—EFFECT OF A SERIES OF CHEMICALS ON THE ISOLATED CULTURE DISK OF TRICHOPHYTON MENTAGROPHYTES

Chemical	60% Polyethylene Glycol 2 Min.	60% Polyethylene Glycol 5 Min.	95% Ethanol 2 Min.
o-Aminophenol	+	+	–
m-Aminophenol	+	+	+
p-Aminophenol	+	+	+
o-Benzylphenol	+	–	–
p-Benzylphenol	+	+	–
Brilliant green	+	–	–
o-Bromophenol	+	+	+
2,4-Dibromophenol	+	+	+
2,4,6-Tribromophenol	–	–	–
2-Br-4-tert-butylphenol	+	+	–
o-Chlorophenol	+	+	+
m-Chlorophenol	+	+	+
p-Chlorophenol	+	+	+
2,4-Dichlorophenol	+	+	+
2,4,6-Trichlorophenol	–	–	–
2,4,5-Trichlorophenol	–	–	–
Chlorothymol	+	–	–
o-Cresol	+	+	+
m-Cresol	+	+	+
p-Cresol	+	+	+
o-Cyclohexylphenol	+	+	–
o-Hydroxybenzaldehyde	+	+	+
p-Hydroxybenzaldehyde	+	+	+
o-Hydroxybenzoic acid	+	+	+
m-Hydroxybenzoic acid	+	+	+
p-Hydroxybenzoic acid	+	+	+
o-Hydroxyphenol	+	+	+
m-Hydroxyphenol	+	+	+
p-Hydroxyphenol	+	+	+
1,2,3-Trihydroxybenzene	+	+	+
1,3,5-Trihydroxybenzene	+	+	+
o-Iodophenol	+	+	+
2,4,6-Triiodophenol (0.75%)	–	–	–
o-Methoxyphenol	+	+	+
m-Methoxyphenol	+	+	+
p-Methoxyphenol	+	+	+
3,5-Dimethylphenol	+	+	+
2,3,5-Trimethylphenol	+	+	+
o-Nitrophenol	+	+	+
m-Nitrophenol	+	+	+
2,4-Dinitrophenol	+	+	–
2,4,6-Trinitrophenol	+	+	+
o-Phenylphenol	+	+	–
p-Phenylphenol	+	+	–
2-Br-4-phenylphenol	+	+	–
Oxyquinoline sulfate	+	+	–
Salicylanilide	+	+	–

understood after performing an agar cup-plate test using 95 per cent ethanol as solvent. At a concentration of 0.1 per cent, the amino compound gives a zone of inhibition with *T. mentagrophytes* of 21 mm. and macroscopically shows that the phenol in the alcohol solution penetrates the agar quickly and for some distance. This must, of course, occur in the disk method of testing, resulting in seemingly high fungicidal activity. Other chemicals such as 2,4-dinitrophenol, 2,4-dinitrocyclohexylphenol, and brilliant green, which stain powerfully, manifest similar results. The disk method using alcohol as a solvent suffers, therefore, from this rapid penetration and inability to remove the penetrated fungicide completely by rinsing, thus permitting an inhibitory substance to exert its effect.

The glycols, particularly the polyethylene types, do not rapidly penetrate the agar disk, which is a distinct advantage. They are not readily reactive chemically, possess negligible toxicity for the ringworm organisms, and have powerful solvent properties. For these reasons, it was originally felt that they provided a suitable medium for fungicidal testing. However, besides their retarding action, other factors might influence their use, such as bonding or association between solvent and test compound. As yet there is no experimental evidence concerning this latter hypothesis.

The ability to penetrate the skin and attack the hyphae and arthropores is essential in order for a preparation to be effective clinically. For this reason many therapeutic preparations contain such volatile and penetrating materials as acetone and alcohol besides surface-active materials. A solvent with good penetrating power would, therefore, more closely simulate this desirable clinical condition for *in vitro* testing. However, it should be borne in mind that such volatile materials do not remain on the skin for any length of time unless compressed there.

The question of solvents for fungicidal and fungistatic testing has yet to be answered satisfactorily.

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Toxicological Studies of Zinc Phosphide*,†

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An investigation has been made to determine some of the acute and chronic effects of zinc phosphide, a rodenticide. Sublethal administration of this compound to domesticated albino rats produces damage to liver and lungs without visible effects on other organs. Evidence is presented to support the assumption that the acute and chronic symptoms of zinc phosphide intoxication are due to phosphine released by gastric acid. Dogs and cats seemed, in general, less susceptible to zinc phosphide than rats, rabbits, and fowls.

For a number of years zinc phosphide (Zn_3P_2) has been used as a poison in rodent control and has proved to be an effective and convenient agent for this purpose. In view of its increasing use, this work was undertaken.

The effects of chronic exposure to zinc phosphide have been studied in albino rats in respect to weight gain, histopathological changes, and fertility. Acute effects in dogs, cats, and rabbits have been noted and a possible mechanism of action for acute and chronic poisoning by zinc phosphide is postulated.

CHRONIC EXPERIMENTS

A preliminary survey indicated that zinc phosphide could be incorporated into a rat's diet at 0.02% and 0.03% levels without undue mortality rates. Some deaths do occur at the higher level.

Exposure.—Rats were individually caged and given water and poisoned bait *ad libitum*. The bait was prepared by grinding the regular ration ("Friskies" brand dry dog pellets), thoroughly incorporating the appropriate percentage of zinc phosphide, and finally, moistening the mixture, shaping into pellets, and air-drying. Rats seemed to waste less food when it is offered in pellet form than when it is offered as a powder.

Series I.—Groups of six albino rats weighing from 70 to 100 Gm. were exposed to baits containing 0.02% and 0.03% zinc phosphide. A control group of like size was kept.

Weight Gains.—Rats were weighed daily, and statistical analyses of weight gains were made after one-week and one-month periods. Rats receiving the 0.03% bait were compared after the one-week period only; these animals failed to gain weight and several soon died. The initial weights of the three groups did not differ significantly. After exposure, however, both experimental groups showed significantly lower gains in weight than the control group. This was true after both the one-week and one-month periods.

Statistical Analysis of Weight Gains.—All rats survived the first week of exposure. After this, however, the 0.03% dosage level proved to be too toxic, and the rats in that group died. Two rats in the 0.02%-level group died, but the remainder survived the experimental period.

Table I presents the initial weights and the weight gains of the control group and of experimental Group I which received 0.03% zinc phosphide. Statistical comparison of the mean initial weights of the two groups shows that the initial groups did not differ significantly ($t = 0.1$). However, the weight gains during one week of exposure were significantly lower in the experimental group ($t = 8.3$).

Table II presents similar data and treatment for experimental Group II which received 0.02% zinc phosphide. In addition to a comparison of the weight gains after one week, another comparison is made after one month. As in Group I, the mean initial weight of experimental Group II did not differ significantly from that of the control group ($t = 0.1$). After one week of exposure, the mean weight gain of the experimental Group II differs significantly from that of the controls ($t = 3.9$).

TABLE I.—COMPARISON OF EXPERIMENTAL GROUP I OF RATS RECEIVING 0.03% ZINC PHOSPHIDE IN DIET WITH CONTROL GROUP

	Rat No.	Initial Weights, Gm.	Gain during One-week Exposure, Gm.
Control Group	C-1	88	25
	C-2	111	35
	C-3	70	26
	C-4	105	26
	C-5	82	28
	C-6	79	24
	Mean:	89.2	26.7
	Standard Deviation of Mean:	±15.7	±4.5
Experimental Group I	E-1	81	8
	E-2	76	8
	E-3	109	3
	E-4	93	2
	E-5	85	11
	E-6	86	−1
	Mean:	88.3	5.2
	Standard Deviation of Mean:	±11.6	±4.5
Mean Difference between Control and Experimental Group I:		0.9	21.5
Standard Deviation of Difference of Two Means:		±8.0	±2.6
Ratio to Standard Error:		0.1	8.3[a]

[a] Significant.

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† Abstracted in part from a thesis submitted to the Graduate Council of the University of Florida by Henry Douglas Johnson in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

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The difference in mean weight gains remains significant after one month of exposure in the experimental group ($t = 3.0$).

Histopathology.—After exposure periods varying from thirteen to thirty-one days in the group receiving 0.03% zinc phosphide and from thirty-nine to fifty-eight days in the group receiving 0.02%, rats were sacrificed by severing the jugular veins. No gross pathology was observed. The following tissues were taken for study: liver, lung, kidney, adrenal gland, intestine, pancreas, spleen, heart, ovary, and testis. Tissue sections were prepared by usual methods using hemotoxylin and eosin stain.

In the liver, several zones of injury were evident, especially about the central and peripheral lobular areas which exhibited injury sufficient to kill the parenchyma cells. For variable distances extending from these areas, the parenchyma cells were disintegrated or in the process of disintegration as manifested by failure of the cell nuclei to stain while the cytoplasm was coagulated and deeply stained with eosin. A great increase in numbers of fibroblastic nuclei was observed within and around the portal canal areas in sections taken from rats receiving more intensive exposure.

The alveolar capillaries of the lung were congested with blood, numerous areas evincing indication of hemorrhage or serous exudation into the alveolar spaces. Considerable mononuclear infiltration was observable around the smaller bronchi and bronchioles of some sections.

No infallible signs of damage were observed in sections of spleen, pancreas, intestine, adrenal, heart, kidney, ovary, or testis.

Series II, Breeding Experiment.—Ten adult male and ten adult female albino rats were exposed to 0.03% zinc phosphide in their diet for twenty-two days. During this period four females and one male died. After removal from the poisoned diet, all animals were bred to normal animals. Each poisoned male was caged with a normal female, and each two poisoned females were caged with one normal male. All poisoned rats proved to be fertile.

ACUTE EXPERIMENTS

Since it has been observed that zinc phosphide seemed nontoxic to animals eating poisoned rats (1), attention was directed to a study of acute effects of zinc phosphide on dogs, cats, and rabbits, as well as rats. Lethal doses of 7–17 mg./Kg. for fowls have been reported, indicating that fowls are quite susceptible to zinc phosphide (2). The oral LD_{50} for rats has been placed at 40.5 ± 2.9 mg./Kg. (3) and its acceptability to them as a poison established (4).

The effects of oral zinc phosphide on blood pressure and respiration were studied in animals anesthetized with sodium pentobarbital I. V. Doses of zinc phosphide were given by stomach tube after recording, on a kymograph, control periods of respiration (via tracheal cannula and tambour) and blood pressure (via carotid cannula and mercury manometer).

Dogs—The results of these experiments with fasting dogs are tabulated in Table III, Part A. They indicate that zinc phosphide alone is not immediately toxic in fasting dogs, even at rather high dosage. It was postulated, however, that this apparent species resistance might be due to a difference in the digestive processes on the poison. A known reaction of zinc phosphide is its release of phosphine (PH_3) in the presence of acids (5). A lack of gastric hydrochloric acid could explain relative immunity to zinc phosphide if the mechanism of its action involved such a release of phosphine. To test this hypothesis, 0.5% hydrochloric acid was given along with zinc phosphide, the results of which are listed in Part B of Table III. Obviously, hydrochloric acid given with zinc phosphide increases the toxicity of the latter in dogs.

TABLE II.—COMPARISON OF EXPERIMENTAL GROUP II OF RATS RECEIVING 0.02% ZINC PHOSPHIDE IN DIET WITH CONTROL GROUP

	Rat No.	Initial Weights, Gm.	Gain during One-week Exposure, Gm.	Gain during One-month Exposure, Gm.
Control Group	C-1	88	25	88
	C-2	111	35	146
	C-3	70	26	122
	C-4	105	22	84
	C-5	82	28	88
	C-6	79	24	88
	Mean:	89.2	26.7	102.7
	Standard Deviation of Mean:	±15.7	±4.5	±25.5
Experimental Group II	E-7	105	3	Died
	E-8	72	6	Died
	E-9	101	22	67
	E-10	98	18	65
	E-11	84	18	64
	E-12	82	7	62
	Mean:	90.3	12.3	64.5
	Standard Deviation of Mean:	±12.9	± 7.9	±2.1
Mean Difference between Control and Experimental Group II:		1.1	14.4	38.2
Standard Deviation of Difference of Two Means:		±8.3	±3.7	±12.8
Ratio to Standard Error:		0.1	3.9*	3.0*

* Significant.

All dogs previously used were fasted. If hydrochloric acid were the necessary factor, then the normal gastric secretion following stimulation by food should make zinc phosphide toxic to dogs. Therefore, 200 mg./Kg. of zinc phosphide was administered in capsules to a fasting intact dog followed by the dog's regular meal. The result is listed in Table III, Part C. Death occurred after

TABLE III.—EFFECTS OF ZINC PHOSPHIDE ON DOGS

Dog No.	Weight, Kg.	Dose Zn_3P_2, Mg./Kg.	Dose 0.5% HCl, Cc.	Time	Observations
		Part A: Anesthetized Dogs			
1 (Female)	12.5	30	..	0:00	No effect
		50	..	3:30	No effect
				5:30	Sacrificed
2 (Female)	11.0	300	..	0:00	No effect
		1,000	..	2:00	No effect
				3:30	Sacrificed
		Part B: Anesthetized Dogs			
3 (Female)	12.0	1,000	75	0:00	Dog vomited, losing part of dose. Blood pressure fell.
				0:45	Respiration stimulated, then depressed. Death.
4 (Female)	7.5	200	40	0:00	Blood pressure depressed; respiration stimulated, then depressed.
				1:00	Death
		Part C: Unanesthetized Dogs			
5		200	..	0:00	Food given along with dose
		(Food also given)		0:43	Vomiting
				3:13	Death

TABLE IV.—EFFECTS OF ZINC PHOSPHIDE ON RABBITS

Rabbit No.	Weight, Kg.	Dose Zn_3P_2, Mg./Kg.	Dose $NaHCO_3$, Gm./Kg.	Time	Observation
		Part A: Anesthetized Rabbits (with $NaHCO_3$, as indicated)			
3 (Male)	2.4	35	...	0:00	No effect
				2:00	Sacrificed
5 (Male)	2.3	35	...	0:00	No effect
				3:00	Sacrificed
1 (Female)	2.75	40	...	0:00	Respiration stimulated, then depressed. (Blood pressure not recorded)
				0:40	Death
6 (Male)	2.8	45	...	0:00	Irregular blood pressure
				2:30	Sacrificed
2 (Female)	2.5	50	...	0:00	Depressed blood pressure
					Respiration stimulated, then depressed
				1:25	Death
		Part B: Anesthetized Rabbits (with $NaHCO_3$, as indicated)			
7 (Male)	2.0	100	0.6	0:00	Irregular blood pressure
				3:30	Sacrificed
Control: 8 (Male)	1.5	100	...	0:00	Falling blood pressure and respiration
				1:10	Death
		Part C: Unanesthetized Rabbits (with $NaHCO_3$, as indicated)			
9 (Female)	1.7	200	0.6	0:00	
				1:00	Death
10 (Male)	2.1	...	0.6	0:00	
		100	...	0:30	Lived over three hours; died during night
11 (Female)	1.3	...	0.6	0:00	
		100	...	0:30	
		...	0.6	1:55	
				5:00	Death
Control: 12 (Female)	1.2	100	...	0:00	
				0:55	Death

three hours and thirteen minutes, showing that normally stimulated gastric hydrochloric acid is sufficient to release toxic amounts of phosphine.

Rabbits.—Effects of zinc phosphide in anesthetized rabbits were obtained in a similar manner. Part A of Table IV contains data for the administration of zinc phosphide alone. It is seen that the toxicity of zinc phosphide to the rabbit is of the same order as to the rat.

In accordance with the hypothesis that stomach acid is an essential factor in the acute effects of zinc phosphide, it was decided to give sodium bicarbonate, along with zinc phosphide, to rabbits in order to ascertain the effect of reduced stomach acid. Part B of Table IV contains the result. Sodium bicarbonate prevented death from a toxic dose of zinc phosphide during the experimental period. To determine the possible value of sodium bicarbonate as an antidote, it was given along with zinc phosphide by stomach tube to intact rabbits. A tabulation is in Table IV, Part C. Concurrent administration of sodium bicarbonate along with zinc phosphide was to no great extent effective in delaying zinc phosphide death. However, administration of sodium bicarbonate thirty minutes ahead of the poison, giving the bicarbonate time to neutralize all acid, delayed death several hours. A repeated dosage with sodium bicarbonate did not save the rabbit's life. This may have been due to ineffective control of stomach acid, or to other toxic mechanisms of zinc phosphide.

Cats.—A similar study was made on cats. Doses of zinc phosphide below 160 mg./Kg. were not effective during the experimental period. Doses from 200 to 300 mg./Kg. gave variable results, death not always following administration. No additional cats were available. However, the data would seem to indicate that cats are less susceptible to zinc phosphide than rats, rabbits, and fowls. Data are presented in Table V.

TABLE V.—EFFECTS OF ZINC PHOSPHIDE ON CATS

Cat No.	Weight, Kg.	Dose Zn_3P_2, Mg./Kg.	Time	Observation
1 (Male)	3.5	50	0:00	No effects
			3:00	Sacrificed
3 (Male)	4.0	120	0:00	No effect
			4:00	Sacrificed
5 (Female)	2.0	160	0:00	No effect
			3:00	Sacrificed
4 (Male)	1.8	200	0:00	Depressed blood pressure
			3:30	Sacrificed
2 (Female)	2.0	250	0:00	Blood pressure depressed
			2:25	Death
6 (Female)	2.3	300	0:00	No effect
			3:00	Sacrificed

Rats.—If, as assumed, the toxicity of zinc phosphide is in part due to the release of phosphine by stomach acid, then poisoned animals should excrete this gas on their breath. This was tested in rats. Two rats (one control, one experimental) were anesthetized with sodium pentobarbital, their tracheas cannulated and connected to one-way valves which forced all exhaled air through vials containing filter paper soaked in 2% silver nitrate solution. Silver nitrate reacts with phosphine to produce a brown-to-black precipitate of elemental silver (5). The experimental rat was given a toxic dose of zinc phosphide via stomach tube. Brown stains first appeared in the vial after fifteen minutes and continued to form until the rat died after one hour and ten minutes. No stains whatsoever appeared on the silver nitrate paper connected with the control rat.

The fact that phosphine is at least partially blown off in the lungs may explain the appearance of pathology in the liver and lungs only in chronically poisoned rats. Phosphine would be absorbed in the intestines, carried to the liver via the portal circulation, then to the lungs via the right heart and pulmonary arteries, and in part blown off. Since in chronically poisoned rats the concentration of zinc phosphide is low, and that of phosphine proportionate, the lungs might effectively remove the phosphine so that organs other than liver and lungs would not be acutely harmed.

The toxicity of phosphine is well established (5). However, for immediate evidence, phosphine was generated by adding hydrochloric acid to zinc phosphide, and the resulting gas led to a bell jar containing a mouse. Death occurred after thirty-five minutes.

DISCUSSION

The release of phosphine by stomach acid as a mechanism for zinc phosphide toxicity is supported by these facts:

(1) One-half per cent hydrochloric acid along with zinc phosphide given orally causes quick death in fasting dogs. Zinc phosphide alone does not cause death during the experimental period even at high dosages.

(2) Similarly, gastric secretion induced by food makes zinc phosphide toxic to dogs.

(3) Sodium bicarbonate delays zinc phosphide death in rabbits.

(4) Poisoned rats excrete phosphine on their breath.

Other facts easily explained by and tending to support this theory are:

(1) Pathology from chronic poisoning is confined to liver and lungs, a fact easily explained by the phosphine release theory.

(2) The relative susceptibility of the rat and the relative immunity of the dog could be explained by the fact that, in the rat, acid secretion is continuous, requiring no stimulus (6). In the dog, secretion is a complex mechanism requiring stimuli and would not be active in a fasting dog.

(3) In fowls it was shown that most of the zinc from zinc phosphide was excreted in the feces and that none was stored in the body (2). This would be quite plausible if phosphine were the toxic agent.

With regard to practical use of zinc phosphide as a rodenticide, the following suggestions may be made concerning danger to animals other than rats: animals likely to eat the carcasses of poisoned rats (dogs and cats) are, in general, more resistant to zinc phosphide than rats. This, plus the fact that they are larger than rats, probably would insure that they would not be fatally poisoned by eating the dead rats. Animals likely to eat the bait itself (rabbits and fowls) are equally or more susceptible to zinc phosphide, and precautions should be taken to pre-

vent their access to the poison. Further investigation of the susceptibility of larger animals to zinc phosphide is indicated.

CONCLUSIONS

(1) Sublethal administration of zinc phosphide to domesticated albino rats produces disintegration of parenchyma cells of the liver accompanied by proliferation of fibroblastic nuclei. Hemorrhagic congestion followed by mononuclear infiltration about the smaller bronchi and bronchioles occurs in the lungs.

(2) Sublethal administration of zinc phosphide has no apparent effect on the spleen, pancreas, heart, intestine, kidney, adrenal, ovary, or testis of the albino rat.

(3) Dogs and cats are, in general, less susceptible to zinc phosphide than are rats, rabbits, and fowls.

(4) Evidence is presented to support the theory that the acute symptoms of zinc phosphide poisoning are due to phosphine released by gastric acid.

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Fungistatic Activity of Antihistamines*

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A number of antihistamines and related compounds have been tested against several fungi. The fungistatic potency does not depend upon their antihistaminic activity. The effect of variations in chemical structure is discussed.

SEVERAL REPORTS have recently appeared (1–4) indicating that antihistamines displayed significant fungistatic activity *in vitro*. Since all of the antihistaminic drugs tested displayed this property, their effectiveness has naturally been attributed to a common ability to antagonize histamine. However, this group of compounds also possesses, in varying degrees, other pharmacological properties (local anesthetic, anticholinergic, antiemetic, etc.). It was of interest, therefore, to test whether a parallelism actually exists between a compound's antihistaminic and fungistatic potencies. In addition, this study affords an opportunity to evaluate the fungistatic action of the antihistaminics now commercially available. Finally, it was hoped that some information might be obtained relating chemical structure to activity.

EXPERIMENTAL

The compounds tested and the manufacturers who made them available to us are listed in Table I. All compounds except Thephorin were dissolved in 70% alcohol and made up to 0.10 *M* solution. Thephorin, because of alcohol insolubility, was dissolved in water and prepared in the same concentration. The test cultures employed were *Tricophyton mentagrophytes*, *Tricophyton* species (Klein), *Microsporum canis*, *Microsporum gypseum*, *Monosporium apiospermum*, *Phialophora verrucosa*, *Sporotrichum Schenkii*, and *Candida albicans*.[1] The method of testing for fungistatic activity was as follows: Sabouraud agar plate surfaces were inoculated with a culture of the test organism. A sterile cotton-tipped swab was dipped into a broth culture of the test fungus and then was used to inoculate the surface of an agar plate. This procedure was repeated, using the same swab, for inoculating each of the test plates with a specific fungus. Filter paper disks[2] impregnated with various concentrations of a test compound were then placed on these inoculated plates. All compounds were prepared so as to have the following solutions for each: 0.10, 0.05, 0.025, 0.01, 0.005, 0.0025, 0.0010, and 0.0005 *M* concentrations. Sufficient disks for each dilution of each

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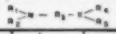
[1] All fungus cultures were obtained from the Fourth Army Area Laboratory, Brooke Army Medical Center, Fort Sam Houston, Tex., with the exception of the *Trichophyton* species (Klein) which was isolated in this laboratory.
[2] Carl Schleicher & Schuell Company, No. 740-E, "Discs for Assay of Penicillin and Other Antibiotics."

TABLE I STRUCTURE OF COMPOUNDS STUDIED



BRAND NAME	GENERIC NAME	MANUFACTURER	R_1	R_2		R_3	X	R_4	R_5
PYRIBENZAMINE	TRIPELENAMINE HCl	CIBA	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$-N<$	$-CH_2-$	
SYNOPEN	CHLORTRIPELENAMINE HCl	GEIGY	CH_3-	CH_3-	$>N-$	$-CH_2CH_2$	$-N<$	$-CH_2-$	
NEOHETRAMINE	THONZYLAMINE HCl	WYETH	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$-N<$	$-CH_2-$	
NEOANTERGAN	PYRAMSAMINE MALEATE	MERCK	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$-N<$	$-CH_2-$	
DIATRINE	METHAPHENILENE HCl	WARNER	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$-N<$		
THENFADIL		WINTHROP	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$-N<$		
HISTADYL THENYLENE	METHAPYRILENE HCl	LILLY ABBOTT	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$=N<$		
TAGATHEN	CHLOROTHENYLPYRAMINE CITRATE	LEDERLE	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$-N<$		
FORALMIN	METHAFURYLENE FUMARATE	EATON	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$-N<$		
TRIMETON	PROPHENPYRIDAMINE MALEATE	SCHERING	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$-\overset{\|}{C}-H$		
CHLORTRIMETON	CHLORPROPHENPYRODAMINE MALEATE	SCHERING	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$-\overset{\|}{C}H-$		
BENADRYL	DIPHENHYDRAMINE HCl	PARKE-DAVIS	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$-O-CH-$		
DRAMAMINE	DIMENHYDRINATE	SEARLE	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$-O-CH-$		
DECAPRYN	DORYLAMINE SUCCINATE	MERRELL	CH_3-	CH_3-	$>N-$	$-CH_2CH_2-$	$-O-\overset{\|}{C}-CH_3$		
LERGIGAN		REID	CH_3-	CH_3-	$>N-$	$-CH_2\overset{\|}{C}H-$ CH_3			
PHENERGAN		WYETH	CH_3-	CH_3-	$>N-$	$-\overset{\|}{C}H-CH_2-$ CH_3			
PARGOL		WARNER	C_6H_5-	C_6H_5-	$>N-$	$-\overset{\|}{C}H-CH_2-$ CH_3			
S-48		PARKE-DAVIS	C_6H_5-	C_6H_5-	$>N-$	$-CH_2CH_2-$	$-O\overset{\|}{C}H-$		
838-C		LEDERLE				$-CH_2CH_2$			
ARYANE		LEDERLE				$-CH_2CH_2$			
47-85	CYCLIZINE HCl	BURROUGHS-WELCOME							
PERAZIL DIPARALENE	CHLORCYCLIZINE HCl	BURROUGHS-WELCOME ABBOTT					$-CH-$		
DIPHENYL PYRALINE		NATIONAL DRUG PRODUCTS					$-O\overset{\|}{C}H-$		
ANTISTINE	ANTAZOLINE HCl	CIBA				$-CH_2$			
PYRROLIZATE	PYRATHIAZINE HCl	UPJOHN				$-CH_2CH_2-$			
ASTEROL		HOFFMAN-LA ROCHE	CH_3-	CH_3-			$-N<$	$-C_6H_5$	$-C_6H_5$
THEPHORIN	PHENINDAMINE TARTRATE	HOFFMAN-LA ROCHE							
SC 2159		SEARLE							
SC 3192		SEARLE							
SC 3171		SEARLE							
SC 3183		SEARLE							
BANTHINE	METHANTHELINE BROMIDE	SEARLE							
ATROPINE									

compound were prepared in order that the effects could be determined for all fungus test organisms.

When the inoculations described above were completed, the sensitivity disks were placed immediately on the surfaces of the agar plates. Two disks impregnated with varying concentrations of the compound to be tested were placed so that any zone of inhibition could be easily noted and measured. A minimum of 4 plates was required for determining the effects of a single compound on one of the test organisms. Results were recorded after six days' incubation at 32° for all fungi with the exception of those for *C. albicans*, which was examined at the end of twenty-four hours.

The zones of inhibition surrounding the antihistamine sensitivity disks were of two types. When no fungus growth was visible in the area surrounding a disk, the zone was called *complete* inhibition. *Partial* inhibition was used to define a zone where minimal growth of the fungus occurred in the area surrounding a sensitivity disk. At the end of the incubation period, the zones of inhibition present on the test culture plates were measured and recorded as to diameter and type for each concentration of the compounds tested.

RESULTS AND DISCUSSION

The effectiveness in completely inhibiting growth of the various fungi is summarized in Table II and the extent of partial inhibition is similarly listed in Table III. Relatively few of the preparations were active against all of the fungi tested. Only Diparalene and Lergigan inhibited completely each of the fungi at concentrations of 0.10 *M* or lower. The *Ph. verrucosa*, *S. Schenkii*, and *C. albicans* were especially resistant, and only a few of the drugs tested showed even partial inhibition at concentrations below 0.10 *M*.

It can readily be seen that there is little correlation between the antihistaminic potency of a compound and its effectiveness as a fungistatic agent. Thus, a number of active antihistaminics failed to have any appreciable action on most of the fungi tested. Decapryn, for example, at the 0.10 *M* level had only a very weak effect on *M. gypseum* and none whatsoever upon the other fungi. Foralmin and Neohetramine were only slightly more effective. Trimeton produced no detectable inhibition whatsoever. On the other hand, the antispasmodic Artane, which has only negligible antihistaminic properties, produced significant inhibition with *T. mentagrophytes*, *Trichophyton* species (Klein), *M. Canis*, and *M. gypseum*. One of the most active preparations (Asterol) has not been reported to possess antihistamine activity.

Since many of the most effective preparations (Lergigan, Phenergan, SC 3192, Parsidol, etc.) strongly inhibit the action of acetylcholine, it was first thought that this pharmacological action might be of importance. Again, however, no correlation could be discerned. Atropine, widely used for its anticholinergic properties, was completely without fungistatic action. Other anticholinergic drugs, Banthine, SC 3171, SC 3183, and SC 2159, were slightly better but were still quite unimpressive. The possibility that both histamine and acetylcholine must be antagonized for inhibition was ruled out when all fungi grew luxuriantly in a mixture of the antihistamine Decapryn with the anticholinergic atropine. At present, therefore, there is no correlation between any known pharmacological properties of the effective drugs and their antifungal action.

Examination of the chemical structures of the preparations tested revealed several interesting relationships. Perhaps the most striking observation was the effectiveness of the phenothiazine derivatives. Lergigan, Phenergan, Parsidol, and Pyrrolozate were all among the most

TABLE II.—MINIMAL MOLAR CONCENTRATION REQUIRED TO INHIBIT FUNGUS GROWTH COMPLETELY[a]

Compound	*T. mentagrophytes*	*T.* Species (Klein)	*M. canis*	*M. gypseum*	*M. apiospermum*	*Ph. verrucosa*	*S. Schenkii*	*C. albicans*
Asterol	0.01	0.05	0.01	0.025	>0.10	>0.10	>0.10	>0.10
Diparalene	0.01	0.05	0.025	0.025	0.05	0.10	0.025	0.05
Lergigan	0.025	0.10	0.025	0.025	0.05	0.10	0.05	0.05
Phenergan	0.025	0.10	0.05	0.05	0.10	>0.10	0.05	0.05
Thephorin	0.05	0.10	0.05	0.10	>0.10	0.05	>0.10	0.10
Diphenylpyraline	0.05	0.10	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10
Parsidol	0.10	0.10	0.10	>0.10	>0.10	>0.10	0.10	0.10
SC 3192	0.10	0.10	0.10	0.10	>0.10	>0.10	>0.10	>0.10
Pyrrolozate	>0.10	0.05	0.10	>0.10	0.10	0.10	0.10	0.10
SC 2159	>0.10	....	0.10[b]	0.10	0.10	0.10	0.10[b]	....
Tagathen	>0.10	>0.10	>0.10	>0.10	>0.10	0.05	>0.10	0.05
Synopen	>0.10	>0.10	0.10	0.10	>0.10	>0.10	>0.10	>0.10
47–83	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10	0.10

[a] The following preparations were inactive with all these fungi at the 0.10 *M* level: Benadryl, Distrin, Neoantergan, Thenylene, Pyribenzamine, Artane, Dramamine, Thenfadil, Chlor Trimeton, Neohetramine, 238-C, Foralmin, Trimeton, Antistine, S-45, Decapryn, Banthine, SC 3171, SC 3183, and Atropine.

[b] Not tested at lower concentration.

TABLE III.—MINIMAL MOLAR CONCENTRATION REQUIRED TO PRODUCE PARTIAL INHIBITION OF FUNGUS GROWTH

Compound	*T. mentagrophytes*	*T.* Species (Klein)	*M. canis*	*M. gypseum*	*M. apiospermum*	*Ph. verrucosa*	*S. Schenkii*	*C. albicans*
Asterol	0.005	0.001	0.0025	0.0025	0.10	0.10	0.10	0.10
Diparalene	0.005	0.01	0.01	0.005	0.05	0.025	0.005	0.05
Lergigan	0.005	0.01	0.01	0.0025	0.05	0.05	0.005	0.05
Phenergan	0.005	0.005	0.01	0.005	0.05	0.10	0.005	0.05
Pyrrolozate	0.01	0.001	0.01	0.0025	0.05	0.025	0.10	0.10
Parsidol	0.01	0.01	0.01	0.0025	0.05	0.025	0.10	0.10
47-83	0.01	0.01	0.01	0.025	0.10	0.05	>0.10	0.10
Diatrine	0.01	0.01	0.025	0.025	>0.10	>0.10	>0.10	>0.10
SC 3192	0.01	0.025	0.05	0.05	0.10	0.10	>0.10	0.10
Synopen	0.025	0.01	0.01	0.005	>0.10	0.10	0.10	>0.10
Artane	0.01	0.01	0.025	0.025	>0.10	>0.10	>0.10	>0.10
Tagathen	0.01	0.05	0.025	0.05	0.10	0.05	>0.10	0.10
Diphenylpyraline	0.025	0.01	0.01	0.01	>0.10	0.10	>0.10	0.10
Thephorin	0.025	0.025	0.025	0.0025	0.10	0.05	>0.10	0.10
Benadryl	0.025	0.025	0.05	0.01	>0.10	>0.10	>0.10	>0.10
S-45	0.05	0.025	0.025	0.05	>0.10	>0.10	>0.10	>0.10
Neoantergan	0.025	0.10	0.10	0.10	>0.10	>0.10	>0.10	>0.10
Chlor-Trimeton	0.025	0.10	>0.10	0.10	>0.10	>0.10	>0.10	>0.10
Dramamine	0.05	0.05	0.025	0.05	>0.10	>0.10	>0.10	>0.10
Thenfadil	0.05	0.10	0.025	0.10	>0.10	>0.10	>0.10	>0.10
Pyribenzamine	0.05	>0.10	0.05	0.10	>0.10	>0.10	>0.10	>0.10
Thenylene	0.10	0.05	0.05	0.10	>0.10	>0.10	>0.10	>0.10
283 C	0.10	0.10	0.10	0.05	>0.10	>0.10	>0.10	>0.10
Antistine	>0.10	>0.10	0.05	0.05	>0.10	>0.10	>0.10	0.05
SC 2159	>0.10	....	0.10[a]	0.10[a]	0.10[a]	0.10[a]	>0.10[a]	
Neohetramine	>0.10	>0.10	>0.10	0.05	>0.10	>0.10	>0.10	>0.10
Foralmin	>0.10	>0.10	>0.10	0.05	>0.10	>0.10	>0.10	>0.10
SC 3171	>0.10	....	>0.10	>0.10	>0.10	>0.10	0.10[a]	....
SC 3183	>0.10	....	0.10	0.10	0.10	0.10	0.10[a]	....
Banthine	>0.10	....	>0.10	>0.10	>0.10	>0.10	0.10[a]	....
Decapryn	>0.10	....	>0.10	0.10[a]	>0.10	>0.10	>0.10	>0.10
Atropine	>0.10	....	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10
Trimeton	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10

[a] Not tried at lower concentration.

effective tested. SC 2159, which contains a phenothiazine nucleus, was the best of the quarternary amines to be tested. However, it was not tested at levels below 0.10 *M*, so no accurate comparison with the antihistamines can be made. Testing a larger series of phenothiazines should be profitable.

Chlorination seems to increase the effectiveness of the compound as a fungistatic agent. In every case examined, the chlorinated compound was more active than its parent. Synopen in 0.05 *M* concentration partially inhibited *M. gypseum*, whereas 0.10 *M* pyribenzamine was required to produce the same effect. Chlor-Trimeton succeeded in inhibiting somewhat *T. mentagrophytes*, *Trichophyton* species (Klein), and *M. gypseum*, while Trimeton was completely ineffective in each instance. Tagathen was more active than Thenylene, and Diparalene was better than preparation 47–83. Comparison of various halogenated relatives is under investigation at present and will be reported shortly.

The substitution of cyclohexane for a benzene group made Artane much more active than 238C against *Trichophyton*, although there was little difference among the other fungi. Similarly, a benzene ring seems to be superior to pyridine. Diatrine being much more active than Thenylene. The substitution of diethylamino group in S-45 for the dimethylamino in Benadryl decreased its effectiveness slightly.

In all cases the variation of activity with structure is suggestive rather than definite, because of the limited number of compounds studied. Further studies along these lines, however, should be of material aid in the synthesis of compounds with increased fungistatic potency.

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The Infrared Drying of Pharmaceutical Extracts*

By S. COLLETT

Infrared drying has been applied to more than forty samples of botanical drugs and extracts. Deterioration during the drying time of fifty minutes was slight, and drying was complete in that period of time.

GENERALLY SPEAKING, a dry extract can be defined as a solution of the active principles of a vegetable drug which has been evaporated to dryness. The preparation of such extracts, especially on the large scale, is by no means such a simple matter as the above definition might imply, however; the thermolabile nature of many drug constituents necessitates that the most careful attention be paid to the conditions under which the evaporation is carried out.

The manufacture of dry extracts usually involves the following stages: (*a*) the extraction of the vegetable drug (in most cases by percolation) with a suitable solvent (almost invariably water, alcohol, or a mixture of the two); (*b*) the evaporation of the resulting aqueous or alcoholic liquor to a soft extract (usually carried out in a vacuum still); (*c*) the drying of the soft extract, to produce a friable solid; (*d*) the reduction of the latter to powder or to granules.

While part (*b*) above can, with very little difficulty, be accomplished at temperatures of 50° or less, the final drying of the soft extract to a solid involves (at least in its later stages) temperatures in the region of 100–115°; and it is here, in stage (*c*) of manufacture, that most damage is done to any thermolabile substances present in the extract. In certain instances, such as belladonna and hyoscyamus extracts, the damage may be assessed by assaying the material for alkaloidal content before and after stage (*c*), since any deterioration due to excessive heat results inevitably in a loss of alkaloid. When dealing with non-alkaloidal extracts, excessive heating during manufacture can almost invariably be detected by determining the proportion of insoluble matter in the finished product and comparing it with that present in the soft extract. In such cases, the dry extract is found to contain a considerably higher percentage of insoluble matter than was present in the intermediate product.

The conversion of soft extract to dry is usually done in a vacuum oven, which generally has hollow steam-heated shelves upon which are placed metal trays, with devices for controlling vacuum and steam pressure, and with inspection windows through which the material may be observed throughout the drying process.

Although it would appear that other methods of drying have been tried, at least on the experimental scale, most of them have received only brief mention in the pharmaceutical literature. The literature does not include any account of any quantitative investigation into the changes brought about in the material being dried by such processes.

During the course of recent researches into the use of infrared radiation in moisture determination, certain chance observations led to the belief that the majority of vegetable extracts would undergo comparatively little deterioration during infrared drying; and it was thought that, if this were the case, a quantitative examination of such extracts before and after drying would yield interesting results.

In consequence, some forty samples, each representing a different batch of extract, were infrared dried on a small scale and the solid products immediately transferred to small airtight containers. Portions of the original soft extract and of the dried product were analyzed with a view to assessing what deterioration had occurred as a result of exposure to infrared radiation.

Details of these experiments and their results are given in the following paragraphs.

EXPERIMENTAL

Undried Soft Extract

Each sample was tested for moisture content and for insoluble matter, as follows:

Moisture.—Five grams of the material was infrared dried to constant weight, the loss in weight being equivalent to the moisture (or volatile matter) present in the original sample. The result was expressed as a percentage.

Insoluble Matter.—Two grams of extract was weighed on a small tared paper and transferred to a weighed centrifuge tube. To it was added 10–15 ml. of the appropriate solvent (i. e., that which was originally employed in the preparation of the extract from the crude drug), and the tube and contents were then carefully warmed by immersion in a steam bath (in the case of alcoholic extracts it was vital that excessive heating be avoided, because of any resulting alteration in alcohol strength). At frequent intervals, the tube was rolled between the hands in order to promote intimate mixing of the warm, softened extract with the solvent and so effect solution. In a few cases it was necessary, owing to the extra-stiff nature of the sample, to stir with a

* Received October 9, 1951, from Sudbury, Suffolk, England.

glass rod. Meanwhile the paper on which the sample was weighed was removed from the tube with the aid of forceps.

When as much as possible of the extract had dissolved, the solution was centrifuged for four to five minutes and the liquid was poured off. Any residue in the tube was washed with an additional 10 ml. of the solvent, centrifuged again for four to five minutes, and the liquid was again poured off.

The tube, with any insoluble residue, was finally dried to constant weight at 100°.

Preparation of Dry Extract

A quantity of extract was spread in an even layer, about $^1/_{16}$ of an inch in thickness, on an aluminum pan. The latter was then placed beneath a 400-watt infrared lamp at a distance of about 12 cm. and left there until the material attained constant weight; i. e., until all moisture had been removed. The resulting brittle mass was immediately removed from the pan, rapidly powdered, and transferred to an airtight container.

Analysis of Product

The infrared dried material was examined for insoluble matter by the same method employed for the soft extract; the initial weighing was accomplished with the greatest possible speed in view of the deliquescent nature of the material.

Results

The results obtained with the various extracts before and after infrared drying are recorded in Table I. In addition to the experimental results, there is recorded for each sample the calculated theoretical insoluble-matter content of the dry extract. This value represents the proportion of insoluble matter which would be present in the dried material if no additional insoluble matter was produced during the drying process, and is calculated from the formula:

$$\text{Theoretical amount insoluble} = \frac{I \times 100}{(100 - M)}$$

where I is the percentage of insoluble matter present in the original soft extract and M is its moisture content.

As will be observed, the choice of material for this investigation was not confined particularly to those extracts which appear in the dry form commercially; the samples were chosen solely with a view to providing as wide a range of drug constituents as possible.

DISCUSSION

Taken as a whole, the results are distinctly encouraging, in that the deterioration resulting from the infrared drying is relatively small in the majority of cases. Out of a total of 43 samples, 28 of them experienced an increase in insoluble matter content of less than 1%, calculated on the dried material.

Moreover, in no case did the drying process bring about any appreciable degree of darkening or other discoloration.

In regard to the physical properties of the dried products, all but two of the latter could be quite easily powdered. As might be expected, however, the powders were all extremely deliquescent; in some cases, even a few seconds' exposure to the atmosphere sufficed to bring about some degree of caking. Storage in nearly full, airtight containers, however, served to prevent any such caking, and under these conditions the powdered extracts retained their free-flowing properties for long periods. This was demonstrated, after completion of analytical tests, by storing the powders in heat-sealed glass ampuls for several months and examining them visually at intervals.

Drying Time and Temperature

Under the conditions outlined, the time required for complete infrared drying varied between thirty and fifty minutes; longer periods would, of course, be necessary if thicker layers of extract were to be dried. Needless to say, that is one of the factors which would call for careful consideration in attempting to step up this mode of drying to the large scale.

The maximum temperature attained during drying was 80°, as compared with maxima of 100–110° in vacuum oven drying. Moreover, it must be remembered that, even in the case of the longest-drying extracts, the material was subjected to 80° for only about one quarter of the total drying time, possibly less.

The Effect on Individual Constituents

In view of the encouraging results obtained in the experiments so far described, it was felt that some further data, illustrating the effect of infrared drying on individual active constituents, would be of interest.

Four of the extracts mentioned in Table I were assayed for their principal constituents; the corresponding infrared dried samples were similarly assayed, and the results compared with "theoretical" values calculated in a manner similar to those appearing in Table I. The alcoholic extracts of kola, licorice, nux vomica, and hydrastis were chosen for this purpose, and the assay methods employed in each case are now briefly outlined.

Determination of Caffeine in Kola Extracts

One gram of sample was mixed with approximately 5 Gm. of calcium hydroxide and 5 Gm. of kieselguhr and the mixture was moistened with water to form a smooth paste. The latter was then carefully heated on a steam bath until converted into a friable mass, which was next exhaustively extracted with hot chloroform.

The chloroformic extract, after filtration, was evaporated to dryness, and the residue dissolved in 2 ml. of fresh chloroform. To this was added 15 ml. of hot water, the mixture was boiled for five minutes, and the hot aqueous solution was filtered into a tared dish. Residue and filter were washed with three 10-ml. portions of hot water which was then added to the tared dish.

The mixed aqueous filtrates were then evaporated to dryness and the residue of caffeine was dried to constant weight at 100°.

The caffeine content was calculated as a percentage.

Determination of Strychnine in Nux Vomica Extracts

The method used was that of the B. P. 1948, 3 Gm. of sample being tested in each case. The B. P.

procedure involves the production of a chloroformic solution of the total alkaloids, removal of the solvent by distillation, treatment of the alkaloidal residue with nitric acid to destroy the brucine, and the extraction of the strychnine (after making alkaline) with fresh chloroform. After removal of the solvent, the alkaloid is finally dissolved in alcohol and estimated titrimetrically.

TABLE I.—RESULTS OBTAINED BEFORE AND AFTER INFRARED DRYING[a]

Extract	Moisture, %	Insol. Matter, % Before Drying	Insol. Matter, % After Drying	Theoretical Amt. Insol., %
Cascara	25.3	0.5	2.4	0.7
Leptandra	18.5	0.4	1.15	0.5
Uvae Ursi (+)	18.8	3.6	3.95	4.4
Buchu (+)	27.0	2.4	5.0	3.3
Cascara (+)	28.7	1.35	3.6	1.9
Cascara	25.0	2.4	4.1	3.2
Licorice	15.6	0.25	2.0	0.29
Licorice (+)	23.5	1.7	2.8	2.2
Convallaria (+)	21.6	2.35	3.1	3.0
Hamamelis	18.8	2.47	5.9	3.0
Hamamelis (+)	26.0	2.25	4.8	3.0
Viburnum	20.2	1.1	1.4	1.37
Valerian	19.8	1.4	9.3	1.75
Gentian (+)	20.0	3.0	4.0	3.7
Coltsfoot (+)	18.6	1.2	3.0	1.4
Kola (+)	20.0	2.6	3.8	3.2
Kola	22.0	1.7	2.5	2.2
Krameria (+)	52.0	9.6	27.1	20.0
Senna fruct (+)	25.2	2.55	3.7	3.4
Hawthorn	10.2	2.5	2.9	2.8
Grindelia	26.0	2.6	4.6	3.45
Horehound (+)	18.8	2.15	3.1	2.6
Cornsilk (+)	16.0	1.8	2.15	2.1
Anthemidis (+)	20.0	1.9	2.65	2.5
Pichi herb (+)	31.6	6.3	9.0	9.1
Burdock	27.5	3.1	4.24	4.27
Foenugreek	22.0	1.2	1.8	1.54
Damiana (+)	40.5	5.1	9.2	8.5
Cascara	17.6	0.75	2.3	0.91
Senna leaf	23.4	0.7	1.1	0.91
Fucus vesic (+)	23.4	2.25	4.0	2.93
Euphorbia	19.0	3.0	3.9	3.7
Sarsaparilla	18.5	1.5	3.5	1.84
Belladonna root	20.0	4.0	6.0	5.0
Belladonna leaf	20.0	3.3	4.9	4.1
Melilotus	18.0	0.75	1.5	0.91
Melilotus	21.5	2.25	4.6	2.86
Nux vomica	20.0	0.5	1.2	0.62
Valerian (+)	22.0	1.3	2.6	1.66
Lupulus	21.0	2.7	4.2	3.4
Bitter orange	18.5	2.6	3.3	3.22
Rheum	22.5	1.8	3.1	2.3
Hydrastis	20.3	0.8	1.4	1.0

[a] Samples marked + were aqueous extracts; remainder were alcoholic extracts.

Determination of Hydrastine in Hydrastis Extracts

Two grams of sample, dissolved in 10 ml. of 70% alcohol, was transferred to a 100-ml. graduated flask; 20 ml. of potassium iodide solution was added, the mixture adjusted to 100 ml. with water, and the flask shaken for several minutes.

A portion of 50 ml. of the mixture was filtered off, made alkaline with ammonia, and extracted by shaking with three separate 30-ml. portions of ether. The mixed ethereal extracts were filtered into a tared flask, the ether was removed by distillation, and the residue was dried to constant weight at 80°. The final weight represents the hydrastine from 1 Gm. of sample.

Determination of Glycyrrhizin in Licorice Extracts

Two grams was dissolved in a few milliliters of water, and the solution was treated with twice its volume of 95% alcohol to precipitate unwanted gummy matter. The liquid was clarified by centrifuging, and the precipitate was washed with a few milliliters of 70% alcohol; the washings were then centrifuged and added to the main liquid.

The mixture was vacuum-distilled to dryness, and the residue was dissolved in a few milliliters of water. The aqueous solution was transferred to a centrifuge tube, twice the volume of dilute sulfuric acid was added, and the mixture was centrifuged. The liquid was poured off from the glycyrrhizin precipitate which was then dissolved in dilute ammonia; the ammonical solution was filtered into a tared dish, evaporated to dryness, and the residue was dried to constant weight at 100°.

Results of Assays

The results obtained from the above assays are summarized in Table II.

It will be observed that the percentage of active constituent lost during drying, calculated in terms of the dried extract, was in no instance greater than 5% (i. e., 5% of that present in the original material); this amply supports the evidence obtained in the earlier experiments that, under the specified conditions, the drying of extracts by means of infrared radiation results in the majority of cases in only a small degree of deterioration, as shown by "insoluble matter" determinations.

It should, perhaps, be emphasized that no attempt was made during this investigation to step up the infrared drying of extracts to the large scale or to solve any of the chemical engineering problems which would inevitably arise.

The author has only a few data relating to the effect of vacuum-oven drying on the insoluble-

TABLE II

Extract	Active Constituent	Before Drying, %	After Drying, %	Theoretical Value, %	Recovery, %
Kola	Caffeine	6.8	8.53	8.7	98
Nux vomica	Strychnine	6.2	7.63	7.75	98.5
Hydrastis	Hydrastine	7.8	9.26	9.75	95
Licorice	Glycyrrhizin	26.5	30.4	31.3	97

TABLE III

Extract	Moisture, %	Initial Insol., %	Final Insol., %	Theoretical Value, %*
Cascara Aqueous	20	2.5	9.2	3.1
Cascara Aqueous	24	2.2	8.1	2.9
Krameria Aqueous	30	3.4	15.7	4.8
Valerian Aqueous	26	4.7	14.3	6.3
Valerian Aqueous	21	1.8	11.6	2.3

* Assuming no increase during drying.

matter content of the product. The small amount of information which is available is summarized in Table III.

SUMMARY

The infrared drying of pharmaceutical extracts has been investigated on the laboratory scale.

More than forty different samples were dried by this means, and the proportion of insoluble matter was determined in the undried and dried material in each case.

By comparing the figures thus obtained before and after drying, it is shown that in the majority of cases the deterioration which occurs during the drying process is slight.

This conclusion is further supported by the results of some additional experiments, carried out on four of the samples, in which the undried and dried extracts were each assayed for a particular active constituent.

None of the samples examined required more than fifty minutes' exposure to the infrared radiation; complete drying was invariably effected within that period.

The maximum temperature reached by the material being dried was 80°.

A Method for the Differentiation of Hydroxocobalamin from Cyanocobalamin Employing the Ascorbic Acid Reaction*

By J. A. CAMPBELL, J. M. McLAUGHLAN, and D. G. CHAPMAN†,‡

The fact that hydroxocobalamin is readily destroyed in solutions containing ascorbic acid while cyanocobalamin is relatively stable in such solutions has been used as the basis of a procedure to determine hydroxocobalamin in the presence of cyanocobalamin. The method is simple, and appears to be specific and reliable.

FOLLOWING earlier work (1, 2) on the reaction of vitamin B_{12} and ascorbic acid, Trenner, *et al.* (3), reported in 1950 that vitamin B_{12a} (hydroxocobalamin) was readily destroyed in solutions containing ascorbic acid, and that vitamin B_{12} (cyanocobalamin) was relatively stable under such conditions. The reaction appeared to be influenced by *p*H.

These data suggested a possible means of differentiating between hydroxocobalamin and cyanocobalamin, using a microbiological assay as the basis for the test. In 1950, Frost, *et al.* (4), reported experience with such a procedure where measurements were made of vitamin B_{12} potency before and after destruction of the vitamin B_{12a} (hydroxocobalamin) with sodium ascorbate. They also described other factors affecting the reaction, but no details of the method were published.

Independent work in this laboratory has led to the development of a somewhat similar method for the differential estimation of vitamins B_{12a} and B_{12b} in the presence of vitamin B_{12}. Vita-

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† The authors are indebted to Dr. H. H. Fricke, Abbott Laboratories, Chicago, and to Dr. G. E. Boxer, Merck & Co., Rahway, N. J., for generous supplies of vitamin B_{12} and B_{12a} respectively.

‡ After this work was completed, the authors were privileged, through the kindness of Dr. D. V. Frost, Abbott Laboratories, to see the draft of a paper outlining experience in their laboratory with this reaction. The data reported here confirm some of their work.

mins B_{12a} and B_{12b} are both hydroxocobalamins and are usually considered to be identical. In this case, they were obtained from different sources. In our laboratory, preliminary work with ascorbic acid confirmed the effect of *p*H on the reaction, and since the stability of vitamins B_{12a} and B_{12b} was greater at a low *p*H, it was decided to use buffers in subsequent investigations. The work reported here was carried out using a sodium acetate (Molar)-HCl buffer at *p*H 5.0.

PROCEDURE

The sample (10–50 μg.) in 1 to 2-cc. solution was added to a large test tube (with pouring lip) containing 150 mg. of ascorbic acid freshly dissolved in 2 ml. of buffer. The tube was then placed in a water bath at 70°. After thirty minutes, the solution was transferred to a volumetric flask and diluted for assay. Microbiological assays for vitamin B_{12} activity were carried out according to the U. S. P. method (5) modified by using a 6-point design on a log dose-log response basis.

RESULTS

This method was applied to solutions of crystalline vitamins B_{12}, B_{12a}, and B_{12b}. The data in Table I indicate that, under the conditions described, complete destruction of vitamin B_{12a} and B_{12b} did not occur until the temperature of the reaction mixture was raised to about 70°. Under the same conditions, vitamin B_{12} was not affected. This was confirmed by absorbancy measurements on the Beckman spectrophotometer at 548 mμ. Table II shows the results of assays of mixtures of vitamins B_{12} and B_{12b}. These mixtures were prepared by combining solutions of the crystalline vitamins in the proportions indicated and applying the ascorbic acid reaction. The recovery of vitamin B_{12} appeared to be unaffected by the amount of B_{12b} present. This constituted further evidence that no measurable amount of the vitamin B_{12b} was present after reaction with ascorbic acid. The precision and accuracy of the method was reasonably good.

TABLE I.—THE EFFECT OF TEMPERATURE ON THE ACTIVITY OF VITAMINS B_{12}, B_{12a}, AND B_{12b} TREATED WITH ASCORBIC ACID FOR THIRTY MINUTES

Temperature, °C	Microbiological Activity Remaining after Treatment, %		
	B_{12}	B_{12a}	B_{12b}
25	...	8.6	8.0
37	...	6.6	3.5
50	99.0	2.0	2.0
60	98.0	...	...
70	98.0	0.1	0.7
75	100.0	...	...

TABLE II.—THE VITAMIN B_{12} ACTIVITY OF MIXTURES OF B_{12} AND B_{12b} AFTER TREATMENT WITH ASCORBIC ACID

Vitamin Added		Vitamin B_{12} Found	
B_{12b}, μg.	B_{12}, μg.	μg.	%
3.8	13.8	14.3	104
5.2	13.8	14.1	102
7.6	9.2	9.0	98
10.4	9.2	9.3	101
11.2	4.6	4.8	104
15.6	4.6	4.5	98

The reliability of the method was also tested by assaying five commercial B_{12} concentrates (fermentation sources) microbiologically both before and after treatment with ascorbic acid and comparing the results with those found using the chemical method of Boxer and Rickards (6). That part of the vitamin B_{12} activity which reacted with ascorbic acid was arbitrarily designated B_{12b}. As shown in Table III, there is good agreement between the proposed microbiological method and the chemical method for the estimation of the vitamin B_{12} content of concentrates. Product B was dark brown in color, which may have prevented the light from penetrating the solution sufficiently to split off all the cyanide in the chemical method, thus giving a recovery too low to estimate. The results indicate that the vitamin B_{12b}/B_{12} ratio varies widely from one concentrate to another and may vary also between different lots of the same product. It appears also that concentrates containing a relatively high proportion of vitamin B_{12} more often meet labeled claim and therefore presumably are more stable preparations.

This procedure has been applied to liver extracts but, as reported by the Abbott workers, iron prevents the destruction of hydroxocobalamin by ascorbic acid. No satisfactory method of removing the iron has been found.

SUMMARY

A procedure has been developed for the differentiation of hydroxocobalamin from cyano-

TABLE III.—THE POTENCY OF VITAMIN B_{12} CONCENTRATES, μG./ML. FERMENTATIVE SOURCE

Company	Lot	Labeled Potency	B_{12} Total Microbiol.	B_{12} (Cyano) Microbiol.	B_{12} (Cyano) Chemical	B_{12b} (Hydroxo)[a]
A	1	20	17.7	2.6	2.6	15.1
B	1	10	6.9	3.4	...	3.5
C	1	30	19.2	12.3	10.5	6.9
	2	30	38.8	2.8	1.4	36.0
D	1	30	38.0	33.3	30.2	4.7
	2	30	35.0	34.8	35.2	0.2
E	1	30	22.0	6.1	6.7	15.9
	2	30	27.6	7.9	7.0	19.7

[a] The difference between microbiological determinations before and after treatment with ascorbic acid.

cobalamin which appears to be specific and reliable and the results compare favorably with those obtained by the chemical assay. To those laboratories which are not equipped for the chemical procedure of Boxer and Rickards, it offers a simple means of detecting the proportions of these vitamins in concentrates from fermentative sources. With liver extracts it has not been found to give effective separation.

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Experiments with Khellin

IV. A Convenient Method for the Preparation of Khellin and Some Observations on the Constituents of *Ammi visnaga* Fruits Grown in Minnesota*

By HAMED ABU-SHADY† and TAITO O. SOINE‡,§

***Ammi visnaga* plants were grown and the unripe fruits were extracted to yield khellin and visnagin. Khellol glucoside was not found in isolable amounts. A modified procedure for the extraction of khellin from *Ammi visnaga* fruits has been developed and appeared to give better yields than previous methods.**

THE FRUITS of *Ammi visnaga* L. contain the closely related furochromones, i. e., khellin,[1] visnagin, and khellol glucoside (1–3) of which khellin is the only constituent possessing a significant medicinal value (4). Khellin has recently been synthesized by Geissman (5) who, however, indicated that his synthetic procedure is not suitable for large-scale production. Therefore, the source of khellin is limited for the time being to the naturally occurring material. Although khellin is not difficult to obtain from the fruits of *Ammi visnaga*, the yields have not been outstanding and the procedures used are somewhat tedious. For this reason, the authors have developed a simplified extraction process which is more convenient and gives excellent yields.

Khellin is ordinarily prepared from *Ammi visnaga* fruits by two principal procedures: (*a*) the ether method, and (*b*) the alcohol method.

(*a*) The ether method (1, 6, 7) involves the extraction of the powdered fruits in a Soxhlet apparatus for about one hundred hours. The ether extract is then concentrated to a small volume and kept in a cool place. The crude mixture of khellin and visnagin (khellol glucoside is insoluble in ether) that separates is removed from the mother liquor by filtration and is freed of fatty substances by washing with ether. Khellin may then be purified by crystallization from boiling water, followed by repeated crystallization from an appropriate organic solvent. The purity and the yield of khellin depend largely on the number of crystallizations. The yields of pure material vary from 0.1 to 0.4 per cent.

(*b*) The alcohol method (8, 9) involves the extraction of the powdered fruits with either hot or cold alcohol, using one of the pharmaceutical processes, i. e., maceration, percolation, or continuous extraction. The alcohol concentration varies from 70 to 95 per cent. The alcoholic extract is concentrated to a small volume and kept in a cool place for a considerable period of time to allow the khellol glucoside to separate. The alcoholic mother liquor, after removal of the

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[1] For an excellent review of khellin and related products, see Huttrer, C. P., and Dale, E., *Chem. Rev.*, **48** 543(1951).

glucoside, is then freed of alcohol and the residue is digested with a large volume of boiling water. The hot, aqueous extract is filtered, cooled, and extracted thoroughly with chloroform. The chloroform extracts are distilled to dryness to obtain a crude mixture of khellin and visnagin which is processed in the same manner described in the ether method. Yields in the neighborhood of 0.3 per cent are usually obtained.

The following procedure for the preparation of khellin, developed by the authors, has proved satisfactory. Powdered *Ammi visnaga* fruits were extracted with Skellysolve-B[2] in a Soxhlet apparatus for about twenty-four hours. Khellin, being sparingly soluble in petroleum ether, separated during the extraction in a relatively pure form and was filtered from the hot extract. The crude product, after being rinsed with Skellysolve-B and crystallized three or four times from alcohol, gave pure khellin, m. p. 153–154°, in 0.8 per cent yield.

Because of a limited supply of *Ammi visnaga* fruits at the initiation of the research, it was decided to attempt the raising of the plants from botanically verified fruits. This attempt was successful. The fruits were germinated in clay pots in March or April in the greenhouse and then transferred to flats. When the plants attained 10 cm. in height, they were transferred to outside plots (May). The plants grew well and exhibited no signs of infections or insect attacks. However, because of the short and relatively cool summers of Minnesota, the fruits were collected in October and early November when they were in a state of maturity but had not yet fully ripened. It is worthy of note that the only other recorded growing experiment with this plant was by Upsher Smith (10) and was also carried out in Minnesota within 50 miles of the plot on which these experiments were conducted. His experiments were termed by him to be successful although the entire plot was wiped out by a grubworm infestation of the roots prior to the fruiting stage.

Extraction experiments on the Minnesota-grown fruits revealed the presence of khellin in exceptionally high percentage together with visnagin in amounts corresponding favorably with earlier findings (2). No isolable amounts of khellol glucoside were found nor were any coumarins present. The lack of significant amounts of khellol glucoside was interesting in view of the fact that it seems to be a constant component of seeds grown in the eastern Mediterranean regions. Whether the high yield of khellin together with an absence of khellol glucoside from the Minnesota-grown *Ammi visnaga* fruits is due to the change in environmental conditions or to collection of the fruits at an unripe stage cannot be decided with the present data.

EXPERIMENTAL

Preparation of Khellin from the Fruits of *Ammi visnaga* L. by the Petroleum Ether (Skellysolve-B) Method.—Five hundred grams of powdered (No. 20) *Ammi visnaga* fruits were packed into a Soxhlet apparatus and extracted for twenty-four hours with Skellysolve-B. As the extraction proceeded, khellin gradually separated, causing slight bumping of the solution. The crystals were filtered from the hot extract with the aid of suction and were washed on the filter with small portions of the solvent. The green-colored crystals (8.4 Gm.) were dissolved in 30 cc. of alcohol with the aid of heat and were filtered through a heated funnel. On cooling, 5.8 Gm. of pale-green crystals, m. p. 148–152°, were obtained. These crystals were dissolved in 25 cc. of alcohol and were refluxed for five minutes with 0.5 Gm. of decolorizing charcoal (Norit). The hot solution was filtered free of charcoal and the filtrate was placed in the refrigerator to crystallize. A crop of slightly yellow crystals (4.7 Gm.), m. p. 151–153°, was obtained, and was recrystallized once more from alcohol to give almost colorless crystalline khellin (4 Gm.), m. p. 153–154°.

Extraction Studies on Minnesota-Grown *Ammi visnaga* Fruits. Material.—The fruits were collected while mature, but not fully ripe, from *Ammi visnaga* plants grown at Rosemount, Minn.[3] Several collections were necessary due to the irregular fruiting of the plants. Collections were made during the months of October and November before the appearance of heavy frosts which damaged the plants seriously. The frost-bitten plants wilted immediately, turned yellow, and ceased to grow. However, a small amount of ripe fruits were collected very late in the season. The fruits were dried in compartments with circulating hot air, freed from other plant fragments as much as possible, and reduced to a No. 20 powder.

The powdered fruits (3,150 Gm.) were extracted for about twenty-four hours in a Soxhlet apparatus, using Skellysolve-B as a solvent. The marc was freed of solvent and reserved for further extraction. The Skellysolve-B extract was then filtered, while hot, from the crystals (solid A, 74 Gm.) that had separated and the crystals were washed with small quantities of the solvent. The washings and the mother liquor were combined and concentrated to 500 cc., and were kept in the refrigerator for a week. A dark-green resinous material separated (solid B) and was removed from the oily extract by decantation. Solid B weighed 41 Gm.

Solid A consisted of green crystals and was dissolved in 250 cc. of alcohol with the aid of heat.

[2] Skellysolve-B is essentially a *n*-hexane fraction, b. p. 63–70°, marketed by the Skelly Oil Co., Solvents Division, Kansas City, Mo.

[3] The first experiments with Minnesota-grown *Ammi visnaga* fruits were conducted on material grown in the summer of 1950 in the College of Pharmacy Medicinal Plant Garden on the University of Minnesota campus. The results were the same as those described for material grown at Rosemount, Minn., in the summer of 1951.

The dark-green solution was filtered while hot. On cooling, 42.5 Gm. of light-green crystals, m. p. 147–152°, were obtained. These were recrystallized three times from alcohol to yield 34.3 Gm. of very pale-yellow prisms, m. p. 153–154°. A mixed melting-point determination of these crystals with an authentic sample of khellin failed to depress the melting point, and the product was thus identified as khellin.

Anal.—Calcd. for $C_{12}H_6O_2(OCH_3)_2$: C, 64.6; H, 4.6; OCH_3, 23.8. Found: C, 64.9; H, 4.5; OCH_3, 24.0.

Solid B was boiled with 2.5 L. of water for fifteen minutes, and the aqueous solution was immediately filtered while hot through a moist filter paper supported at the bottom by a pad of cotton gauze. The filtered solution was allowed to stand in the refrigerator overnight. White filiform crystals (solid C, 10.5 Gm.), m. p. 115–123°, were obtained and removed by filtration. The aqueous mother liquor was transferred to a separatory funnel and extracted repeatedly with small portions of ether. The combined ether extracts were dried over anhydrous sodium sulfate and concentrated to a small volume. When this concentrated extract was cooled in the refrigerator, it yielded 1.5 Gm. of colorless, prismatic crystals, m. p. 150–153°, which were found to be identical with khellin. An additional quantity of khellin (1.2 Gm.) was obtained by further working up the ethereal mother liquor.

Solid C was dissolved in 50 cc. of ether, filtered, and treated with enough petroleum ether (b. p. 30–60°) to cause turbidity. On standing in the refrigerator, a crop of 4.3 Gm. of crystals was obtained and subsequently identified as khellin. The filtrate from these crystals was treated with more petroleum ether and, on cooling again, a 3.5 Gm. crop of colorless crystals was obtained, m. p. 130–136°. These crystals were recrystallized three times from a mixture of ether and petroleum ether to yield 2.2 Gm. of product, m. p. 143–145°. A mixed melting-point determination with authentic visnagin showed no depression and the product was identified as visnagin.

Anal.—Calcd. for $C_{12}H_7O_2(OCH_3)$: C, 67.8; H, 4.3; OCH_3 13.5. Found: C, 68.1; H, 4.2; OCH_3 14.0.

The marc was exhausted by percolation with alcohol. Most of the alcohol was removed from the percolate under reduced pressure and the residual extract was kept in the refrigerator for two weeks. A brown syrup separated but no crystals were obtained. The syrupy material was sparingly soluble in alcohol but was completely soluble in cold water. Its sweet taste suggested a carbohydrate nature. When crystallized from alcohol, it yielded colorless, sweet-tasting prisms, m. p. 179° with foaming and decomposition. These crystals failed to reduce Fehling's solution until hydrolyzed with hydrochloric acid, but were not investigated further.

The alcohol was then removed completely from the extract, following the removal of the saccharine syrup, to leave a soft, dark-green mass. This residue was boiled with 5 L. of water and filtered while hot. A dark-red aqueous filtrate was obtained which was cooled and transferred to a separatory funnel where it was extracted with several small volumes of chloroform. The chloroformic extracts were combined, washed with water, dried over anhydrous sodium sulfate, and distilled. The residue crystallized on cooling. Purification of these crystals by crystallization from alcohol yielded 4.2 Gm. of khellin, identified by a mixed melting-point determination. The chloroform-extracted aqueous filtrate was freed of chloroform by heating on a steam bath, and then was extracted completely with small portions of ethyl acetate. The combined ethyl acetate extracts were washed, dried, and then concentrated. A yellow, phenolic, amorphous substance separated on cooling but resisted all attempts at crystallization. Other trials directed toward the isolation of khellol glucoside, representing modifications of the above procedure, were also unsuccessful.

SUMMARY

1. A convenient method for the preparation of khellin using Skellysolve-B as a solvent has been described. The method is not only less tedious than those previously used but also appears to give better yields than have heretofore been experienced.

2. The constituents of Minnesota-grown *Ammi visnaga* fruits collected at a mature but unripe stage have been investigated. Khellin and visnagin were found to be present in the fruits, but no khellol glucoside could be isolated although several attempts were made to do so. This finding was unique in that the same plant when grown in the eastern Mediterranean regions has khellol glucoside as a constant component.

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The Anhidrotic Action of Atropine on Human Thermoregulatory Sweating*

By MORTON J. RODMAN†

The extent to which thermally induced sweating is reduced by various dose levels of atropine was measured. Large doses did not inhibit sweating to a significantly greater degree than did the smallest dose employed. The peripheral depression of sweat gland neuroeffectors by atropine is antagonized by the effects of larger doses of atropine upon various other sites of action.

THE DOSES of atropine employed clinically in hyperhidrosis appear to have been arrived at empirically, as the literature contains no quantitative studies of the effect of various dose levels of atropine upon the amount of sweat secreted by humans under varying physical and environmental conditions.

The purpose of this study was the measurement of the relative efficiency of various dose levels of atropine in inhibiting thermally induced sweating and the determination of the extent to which the peripheral action of atropine may be modified by the effect of atropine on centrally located portions of the sweat mechanisms and on other related systems.

METHODS AND PROCEDURES

Six young male subjects were exposed to temperatures of 104° F. dry bulb/87° F. wet bulb and 113° F. dry bulb/87° F. wet bulb in a constant temperature and humidity chamber. (Several tests were also made at 122° F. dry bulb/87° F. wet bulb but the effects of this temperature upon subjects who ingested large doses of atropine were so severe that it was decided to abandon this portion of the experiment.) A minimum of nine control tests was made at each temperature over a period of several weeks in order to establish a normal mean sweating rate for each subject. During the same period, three doses of atropine sulfate (0.6 mg., 1.2 mg., and 2.4 mg.) were each administered at least three times to all subjects one hour before they underwent the period of high heat. The mean sweating rates after pretreatment with the various doses of atropine were compared with the normal mean sweating rate in order to determine the degree of inhibition of sweating brought about by each dose. Changes in pulse rate and body temperature were also measured. Reactions to the drug and to heat were observed and recorded.

Sweat was collected from two bilaterally symmetrical areas of abdominal skin to which were taped previously weighed plastic capsules 54 mm. in diameter, containing highly absorbent cotton pads. The added weight of the capsules at the end of the one-hour period represented the sweat secreted in the circumscribed area during that time. This local sweating was assumed to reflect the sweating rate for the entire body, in accordance with Kuno's (1) experimental proofs that conclusions as to general sweating may be drawn from the measurement of sweating in small regions of the skin.

In accordance with earlier experiences in this laboratory and with the observations of Eichna, *et al.* (2), and of Adolph (3), a regimen was established which would minimize the factors that tend to produce variability in sweat secretion during prolonged experiments.

RESULTS

Table I and Figs. 1, 2, and 3 summarize the data obtained from all six subjects indicating the mean amounts of sweat collected, mean body temperatures, and mean pulse rates under normal conditions and after exposure to heat and to ingestion of various doses of atropine.

Effect of Heat and of Atropine upon Rates of Sweating.—At both temperatures the rates of sweating showed the wide range of individual variation which is invariably reported when the sweat rates of any group are measured. However, in all individuals, the effect of the several doses of atropine upon sweat secretion was substantially the same as that indicated for the group as a whole.

The smallest dose of atropine employed (0.6 mg.) caused an average reduction in sweating of approximately 41% under both sets of environmental conditions. The two larger doses of atropine (1.2 mg. and 2.4 mg.) reduced sweating only very slightly more than did the smallest dose at each temperature. In a few instances in some individuals, the mean sweating rate was actually slightly greater after the higher doses of atropine than after the smallest dose. Such results, though occurring only occasionally, tended to emphasize the fact that marked increases in the dose of atropine did not exert a greater anhidrotic action than was obtainable with a relatively small dose.

Effect of Heat and of Atropine upon Pulse Rates.—Exposure to heat for one hour at each of the high temperatures employed caused a slight increase in pulse rate (about 10 b. p. m.), when no drug had been previously administered.

The slight slowing of the pulse (2–5 b. p. m.) caused by the two smaller doses of atropine at room temperature was converted to a moderate rise in rate (about 15–25 b. p. m.) during one hour of heat stress. The largest dose of atropine, which at room temperature had caused only a slight rise (about 10 b. p. m.) in pulse rate, brought about a great acceleration of the heart rate during an hour of heat. In some individuals, the pulse rate rose from about

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TABLE I.—GROUP MEANS. EFFECT OF SEVERAL DOSE LEVELS OF ATROPINE UPON PULSE RATE, BODY TEMPERATURE, AND THERMAL SWEATING

Dose Atropine Sulfate, mg.	Mean Sweat, Gm./54 mm. Disk	Mean Body Temp. before Heat	Mean Body Temp. after Heat (1 hr.)	Mean Pulse Rate before Heat	Mean Pulse Rate after Heat (1 hr.)
	Measured at 104° F. Dry Bulb/87° F. Wet Bulb				
Control	0.478	98.4°	98.9°	82	91
0.6	0.280	98.3°	98.9°	78	97
1.2	0.296	98.3°	99.2°	80	115
2.4	0.259	98.4°	99.6°	91	135
	Measured at 113° F. Dry Bulb/87° F. Wet Bulb				
Control	0.706	98.4°	99.1°	82	92
0.6	0.411	98.3°	98.9°	78	100
1.2	0.365	98.3°	99.2°	80	118
2.4	0.357	98.4°	99.8°	91	141

80 b. p. m. to over 160 b. p. m. during one hour of heat.

Increases in heart rate so far above those brought about by the drug alone or by heat alone were probably due to a synergistic effect. Cardiac acceleration caused by depression of the peripheral vagus by larger doses of atropine were potentiated by the stimulating effect of heat upon the metabolic activity of the sinoauricular node and the conducting tissues of the heart.

Effects of Heat and of Atropine on Body Temperature.—Although hyperthermia has been described from moderate doses of atropine administered to children, and larger doses have resulted in a phenomenon called "atropine fever," seen occasionally in infants (4, 5), none of the doses employed in these experiments caused any significant effects on body temperature under normal environmental conditions. The body temperature of all subjects remained essentially the same one hour after administration of each dose level of atropine.

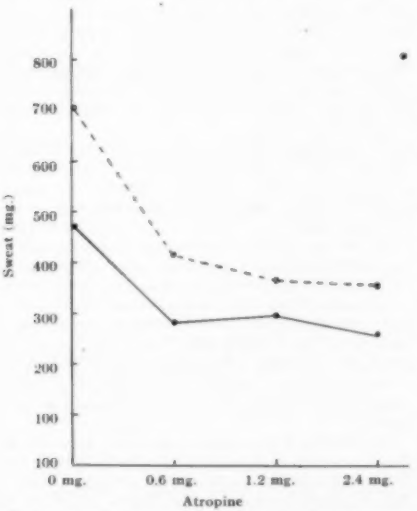

Fig. 1.—Group mean sweat rates. Mean amounts of sweat obtained (54 mm. diameter disk) during hour at 104° F. D. B. and 113° F. D. B. without $\frac{104°}{87°}$ F. W. B. $\frac{113°}{87°}$ F. W. B. atropine and after each of three doses of atropine sulfate. -O-O-O- 113° F. D. B. / 87° F. W. B. —●-●-●- 104° F. D. B. / 87° F. W. B.

After the smallest dose of atropine (0.6 mg.), the body temperature was not increased by one hour of heat beyond the moderate increase brought about by heat alone. With the larger doses of atropine, however, the body temperature tended to rise markedly during the period of heat. This was especially true of the highest dose of atropine (2.4 mg.), which caused a sharp rise in the body temperatures of all individuals during an hour of heat in the cabinet. The rise in body temperature averaged 1.4° F., and in some instances the increase was nearly 2° F.

Increased body temperature after atropine has been attributed in the past entirely to its anhidrotic action. Suppression of sweating has been thought to bring about diminished ability of the body to lose heat by evaporation of water from the surface of the skin. Although this is undoubtedly true, it can not account entirely for the rises in temperature seen in this study, because sweating was not inhibited to a significantly greater extent by the larger doses of atropine than by the smaller doses, while, as has been shown, such smaller doses had very little effect on body temperature beyond the rise caused by heat alone.

The steep rise in body temperature, which occurs in response to high heat after the larger doses of atropine, probably stimulates the sweat centers in the anterior portion of the hypothalamus, which would result in an increased number of impulses from the centers to the sweat glands, thus tending to antagonize the peripheral depression of the glands by atropine.

Effects of Atropine and of Heat upon Comfort and Physical Condition.—In all subjects, one hour of heat alone and of heat together with a dose of 0.6 mg. of atropine produced no discomfort and no unusual subjective or objective effects.

Atropine in doses of 1.2 mg. caused dryness of the mouth and throat, together with thirst. After only a few minutes of heat, a marked flushing of the skin made its appearance in the "blush area" almost invariably. There was rarely any feeling of illness from this dose while the subject was in the cabinet, and the increased pulse rate returned rap-

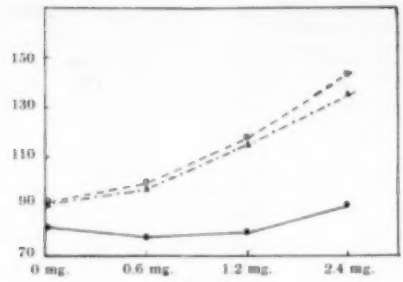

Fig. 2.—Group mean pulse rate.—●—●—Room temp.; ——○ - ○——104° F. D. B. 87° F. W. B. —△—△— 113° F. D. B. 87° F. W. B.

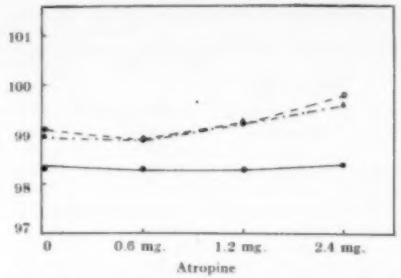

Fig. 3.—Group mean body temperature.—●—● Room temp.; ○—○—104° F. D. B. 87° F. W. B. —△—△—113° F. D. B. 87° F. W. B.

idly to nearly normal at room temperature. Except for increased dryness of the throat, no other uncomfortable symptoms due to atropine persisted or appeared during the remainder of the afternoon.

With the largest dose of atropine (2.4 mg.) a few minutes of high heat produced a very fast, rather shallow pulse rate and a scarlet flush in the "blush area." Occasionally, dizziness, nausea, and headache occurred, and on the few occasions when a temperature of 122° F./87° F. was employed, the usually high-spirited, good-humored group of young men became short-tempered and easily annoyed with one another. After leaving the cabinet, this irritation and the more acute symptoms of illness shortly vanished. However, for several hours afterward, the mouth and throat were uncomfortably dry and the subjects frequently complained of listlessness, drowsiness, and the development of a throbbing headache. Very often dilation of the pupils and some blurring of vision occurred, which made reading difficult, and which, together with slight motor incoördination, interfered with the ability of the subjects to do close mechanical work.

DISCUSSION

The anhidrotic action of the parasympathetic, nervous-system depressant, atropine, on the sympathetically innervated sweat glands was a pharmacological anomaly, until Dale and Feldberg (6) demonstrated the cholinergic nature of the sweat gland innervation in 1934 and thus clarified the responses of the sweat glands to parasympathomimetic drugs. The potent inhibitory action of atropine on the sweat glands is now believed to be due to the antagonism of acetylcholine released in the effector cells of the sweat glands by cholinergic impulses. The extent to which this peripheral action of atropine upon the sweat glands might be antagonized or otherwise affected by the action of atropine upon other parts of the sweat mechanism and upon other systems does not appear to have been investigated.

The results of these experiments indicate that the effectiveness of atropine in reducing thermally stimulated sweating may be self-limited by its effects on sites of action other than the sweat glands. The inhibition of sweating by the depressant action of atropine, even in small doses, upon the effector cells of the sweat glands seems to be antagonized by the complex actions of atropine, especially in higher doses, upon centrally located parts of the sweat apparatus and elsewhere. The data on pulse rates and body temperatures obtained in these tests, together with the observation of central nervous system actions, lend support to the conclusion that atropine directly or indirectly produces effects which tend to neutralize the peripheral depression of the sweat glands.

Direct stimulation of the thermoregulatory centers by chemical agents was demonstrated by Cushing (7). The steep rise in body temperature in response to high heat after the larger doses of atropine may be the result of such a direct stimulation of hypothalamic centers by atropine, which is known to stimulate medullary and higher cerebral centers in larger doses.

The synergistic effects of atropine and of heat upon the systemic circulation and that of the skin result in an increased flow of blood through the skin, a condition which usually accompanies profuse and prolonged sweating. Thus, the sharp rise in pulse rate and the scarlet flush observed on exposure to heat after the larger doses of atropine are indicative of conditions favorable to sweating. Such conditions produced by the action of atropine on the central nervous system and circulatory systems account in part for the "ceiling effect" or limited ability of atropine to reduce sweating by its peripheral depressant action, despite administration of doses large enough to produce minor symptoms of atropine toxicity.

The failure of even toxic doses of atropine to suppress sweating completely may also indicate a possible noncholinergic component in sweating. Haimovici (8), Sonnenschein (9), and Wada (10) have recently presented evidence that at least some sweat glands in certain individuals are sensitive to the direct action of epinephrine. The distribution of such epinephrine-sensitive sweat glands and their relationship to thermoregulatory and emotional sweating is under investigation. Such an adrenergic component, if it were to be proved of importance in thermal sweating, might account for the observed failure of atropine to inhibit sweating completely.

as the anticholinergic action of atropine would, of course, be ineffective against an epinephrine-like compound released at sweat gland neuroeffectors.

Another possible noncholinergic stimulus to sweating has recently been demonstrated by Randall (11), who presented evidence of the ability of the sweat glands to respond directly to intense radiant heat even when reflex impulses were entirely blocked by atropine iontophoresis. However, it is unlikely that such direct response of the sweat glands could have been elicited by the less severe heat employed in our own tests.

The possibility that the moisture obtained, even after large doses of atropine, might have been due to water vapor passing through portions of the epidermis other than the sweat glands may be discounted. Moisture lost by such passive physical diffusion comprises only a comparatiyely tiny fraction of water vaporized from the skin at high temperatures.

SUMMARY AND CONCLUSIONS

1. Thermally stimulated sweating was reduced about 40–50% by a small dose of atropine (0.6 mg.).

2. The administration of two and four times this dose of atropine brought about only a relatively slight further reduction in sweating, while causing a marked rise in pulse rate and body temperature.

3. Several possible mechanisms by which the anhidrotic action of atropine may be antagonized by other atropine effects, which act as a stimulus to sweating, are discussed.

4. It may be concluded that relatively small oral doses of atropine may be as effective as much larger doses for controlling excessive sweat responses, while having the advantage of freedom from annoying side effects.

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Comparative Potency of *d*-Tubocurarine Chloride U. S. P. and Dimethyl Ether *d*-Tubocurarine Iodide*,†

By E. E. SWANSON, W. R. GIBSON, and C. E. POWELL

Metubine iodide[‡] was compared with *d*-tubocurarine chloride in rabbits, rats, and mice. Tests were also carried out on intact and isolated gastrocnemius muscle of rats, isolated diaphragm phrenic nerve of rats, and isolated rectus abdominus of frogs. With the exception of the mouse and the rectus abdominus of frogs, Metubine iodide was found to be many times more potent than *d*-tubocurarine chloride.

In a previous communication (1) the pharmacology of dimethyl ether *d*-tubocurarine iodide was compared to *d*-tubocurarine chloride. The methylated alkaloid inhibited electrically induced contractions of the gastrocnemius muscle of the rat and cat. It reduced the effect of acetylcholine on the frog's rectus muscle. Compared with *d*-tubocurarine, it was close to eight times as potent, the rabbit head-drop method being used. The duration of action was longer with the methylated derivative in the rabbit and cat than with *d*-tubocurarine.

The present investigation is concerned with the potency of dimethyl ether *d*-tubocurarine iodide by a number of known biological methods in comparison with *d*-tubocurarine chloride.

The methods used were: (*a*) U. S. P. head-drop method in rabbits (2, 3) and the single dose cross-over method in rabbits; (*b*) sloping screen method in rats by Allmark and Bachinski (4)

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‡ Registered trade mark of Eli Lilly & Co. for dimethyl tubocurarine iodide.

and in mice by Skinner and Young (5, 6); (*c*) intact gastrocnemius-sciatic nerve modified technique in rats by Van Maanen (7) and isolated gastrocnemius-sciatic nerve method in frogs; (*d*) Bülbring's method (8–11), the isolated phrenic nerve-diaphragm preparation in rats; and (*e*) de Jaloń's method (12), the isolated rectus abdominus muscle of frogs. Actually, eight methods were employed.

For the head-drop methods, a total of 100 rabbits was repeatedly used at bi-weekly intervals. These rabbits were previously conditioned according to the U. S. P. XIV technique. The weights of the rabbits varied from 2,520 to 3,775 Gm. (an average of 2,850 Gm.). For each assay by the U. S. P. head-drop method, the lowest number of rabbits used was 18 and the highest was 28. In the single dose cross-over head-drop method, each assay took a total of 18 to 30 rabbits.

For the sloping screen method, a total of 585 rats and 640 mice was used. A patterned distribution of their weights was used for each assay. The rats varied in weight from 120 to 255 Gm. (an average of 178.7 Gm.). The weights of the mice varied from 12 to 38 Gm. (an average of 22.9 Gm.). Each assay required 30 to 60 rats and 40 to 60 mice. Both rats and mice were injected subcutaneously over the abdominal area.

For the intact gastrocnemius-sciatic nerve (electrically induced contractions), a total of 56 rats was used. Their weights varied from 210 to 275 Gm. (an average of 244 Gm.). The drugs were administered by jugular vein. For the isolated gastrocnemius-sciatic preparation, a total of 15 frogs was used and their weights averaged 60 Gm. A total of 25 rats was employed for the isolated phrenic nerve-diaphragm method. Their weights varied from 375 to 475 Gm. (an average of 410 Gm.). Twenty-five isolated frogs' rectus abdominus muscles were studied, their weight variations being 40 to 90 Gm. (an average of 80.7 Gm.). The isolated muscle preparations required special bath chambers, but no temperature control was necessary. In fact, the isolated phrenic nerve-diaphragm method was just as accurate, and lasted longer, when maintained at room temperature.

The eight samples of methylated *d*-tubocurarine iodide employed in the present work were air-dried and were used in the form of trihydrate; each lot melted at 267–270° with decomposition and each had a specific rotation of $[\alpha]_D^{25} + 150°$ in aqueous solution.

All samples were readily soluble in water. In all cases each lot of methylated alkaloid was compared with the U. S. P. *d*-tubocurarine chloride in the form of pentahydrate.

EXPERIMENTAL

Rabbit Head-Drop Methods.—As shown in Table I, in comparison with U. S. P. *d*-tubocurarine, the U. S. P. method for the eight samples of dimethyl ether derivative required a geometric mean of 0.1108 ± 0.0041 mg. for each 1 mg. of U. S. P. *d*-tubocurarine. Therefore, the methylated alkaloid was 9.02 ± 0.33 times as potent as U. S. P. *d*-tubocurarine.

With the single dose injection of the dimethyl ether alkaloids, a geometric mean of 0.0166 ± 0.0010 mg./Kg. was required to produce the head-drop, and 0.137 ± 0.004 mg./Kg. of U. S. P. *d*-tubocurarine was required to produce the same effect. Thus, the methylated alkaloid is 8.25 ± 0.55 times as active as the standard *d*-tubocurarine. The two methods yielded potency figures for the two alkaloids without significant differences. The single dose cross-over method, however, consumes less time. The technique is more simple and less injurious to the rabbit's marginal ear vein, although a few more rabbits may be needed for each test. The latter method was used for more than three years in this laboratory prior to the adoption of the U. S. P. method.

Sloping Screen Method.—As shown in Table II, *d*-tubocurarine in rats showed a mean (geometric) of

TABLE I.—COMPARATIVE POTENCY OF *d*-TUBOCURARINE CHLORIDE U. S. P. AND DIMETHYL ETHER *d*-TUBOCURARINE IODIDE IN RABBITS, ADMINISTERED BY VEIN

Drug	Sample	U. S. P. Method: Dose, Mg.	U. S. P. Method: Geometric Mean ± S. E., Mg.[a]	Single Dose Injection Method: Dose, Mg./Kg.	Single Dose Injection Method: Geometric Mean ± S. E., Mg./Kg.[b]
d-Tubocurarine chloride	U. S. P. standard	.....	1	.....	0.137 ± 0.004
Dimethyl ether *d*-tubocurarine iodide	1	0.1057 ± 0.0040	0.1108 ± 0.0041	0.0171 ± 0.0007	0.0166 ± 0.0010
	2	0.0955 ± 0.0029		0.0161 ± 0.0010	
	3	0.1156 ± 0.0052		0.0159 ± 0.0010	
	4	0.112 ± 0.0045		0.0187 ± 0.0012	
	5	0.125 ± 0.0048		0.0163 ± 0.0011	
	6	0.113 ± 0.0045		0.0158 ± 0.0010	
	7	0.111 ± 0.0033		0.0161 ± 0.0010	
	8	0.115 ± 0.0040		0.0170 ± 0.0007	

[a] Metubine iodide (dimethyl tubocurarine iodide, Lilly) is 9.02 ± 0.33 times as potent as *d*-tubocurarine chloride by the U. S. P. method.

[b] Metubine iodide (dimethyl tubocurarine iodide, Lilly) is 8.25 ± 0.50 times as potent as *d*-tubocurarine chloride by the single dose injection method.

TABLE II.—COMPARATIVE POTENCY OF *d*-TUBOCURARINE CHLORIDE U. S. P. AND DIMETHYL ETHER *d*-TUBOCURARINE IODIDE BY THE SLOPING SCREEN METHOD

Drug	Sample	Rats, Subcutaneously: Dose, Mg./Kg.	Rats, Subcutaneously: Geometric Mean ± S. E., Mg./Kg.[a]	Mice, Subcutaneously: Dose, Mg./Kg.	Mice, Subcutaneously: Geometric Mean ± S. E., Mg./Kg.[b]
d-Tubocurarine chloride	U. S. P. Standard	.....	0.0897 ± 0.0075	.....	0.2410 ± 0.012
Dimethyl ether *d*-tubocurarine iodide	1	0.0116 ± 0.0049	0.01115 ± 0.00051	0.344 ± 0.0179	0.3001 ± 0.0176
	2	0.0110 ± 0.0057		0.296 ± 0.0207	
	3	0.0101 ± 0.0046		0.311 ± 0.0152	
	4	0.0119 ± 0.0045		0.379 ± 0.0216	
	5	0.0103 ± 0.0030		0.263 ± 0.0126	
	6	0.0104 ± 0.0045		0.306 ± 0.0197	
	7	0.0118 ± 0.0047		0.230 ± 0.0164	
	8	0.0134 ± 0.0090		0.284 ± 0.0150	

[a] Metubine iodide (dimethyl tubocurarine iodide, Lilly) is 8.04 ± 0.33 times as potent as *d*-tubocurarine chloride in rats.
[b] Metubine iodide (dimethyl tubocurarine iodide, Lilly) is 0.803 ± 0.048 times as potent as *d*-tubocurarine chloride in mice.

0.0897 ± 0.0075 mg./Kg. for paralysis. The dose for the methylated alkaloid varied from 0.0101 ± 0.0046 to 0.0134 ± 0.009 with a mean of 0.01115 ± 0.0051 mg./Kg. for the eight samples. The methylated derivative, therefore, is 8.04 ± 0.33 times as potent as *d*-tubocurarine in rats by the sloping screen paralysis method.

In mice, however, the dose of *d*-tubocurarine and dimethyl ether derivative was much larger, as compared to that for the rat. The average dose for *d*-tubocurarine to cause paralysis was 0.241 ± 0.012 mg./Kg. The eight samples of the methylated alkaloid gave a mean of 0.3001 ± 0.0176 mg./Kg.; thus the methylated alkaloid is only 0.803 ± 0.048 times as active as *d*-tubocurarine. When molar equivalent solutions of *d*-tubocurarine and the methylated derivative were injected into mice, the two alkaloids were found to be equal in potency.

Gastrocnemius-Sciatic Method.—With the modified intact gastrocnemius-sciatic method, as shown in Table III and Fig. 1, a dose of 0.05 mg./Kg. of *d*-tubocurarine by vein produced an inhibition of electrically induced contractions of the muscle, equal to 76.3 ± 3.60% of normal. The methylated derivative showed an inhibition of 74.39 ± 6.46% with a dose of 0.01 mg./Kg.; thus the methylated alkaloid is approximately five times as active as *d*-tubocurarine on the gastrocnemius muscle of rats.

In the frog's isolated gastrocnemius-sciatic preparation, the amount required to produce approxi-

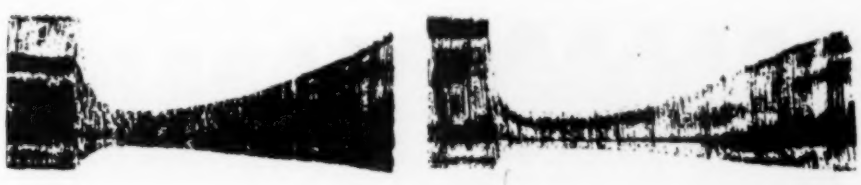

Fig. 1.—The intact gastrocnemius-sciatic nerve of a rat with electrically induced contractions. 50 μg./Kg. of *d*-tubocurarine produced the same inhibition of muscle as 10 μg./Kg. of the methylated alkaloid.

TABLE III.—COMPARATIVE ACTIVITY OF *d*-TUBOCURARINE CHLORIDE U. S. P. AND DIMETHYL ETHER *d* TUBOCURARINE IODIDE BY THE INTACT AND ISOLATED GASTROCNEMIUS-SCIATIC NERVE METHODS

Drug	Sample	Rats, Administered by Vein: Dose, Mg./Kg.	Rats, Administered by Vein: Inhibition, %	Rats, Administered by Vein: Geometric Mean ± S. E., %[a]	Frogs: Dose, Mg.	Frogs: Inhibition, %	Frogs: Geometric Mean ± S. E., %[b]
d-Tubocurarine chloride	U. S. P. Standard	0.050	.....	76.3 ± 3.60	20	.....	94.2 ± 4.5
Dimethyl ether *d*-tubocurarine iodide	1	0.010	78.63 ± 8.41	74.39 ± 6.46	5	92.5 ± 3.5	93.89 ± 4.14
	2		78.63 ± 8.40				
	3		80.79 ± 5.45			95.2 ± 4.5	
	4		78.58 ± 6.78				
	5		71.99 ± 9.29			92.4 ± 3.5	
	6		78.91 ± 5.79				
	7		66.95 ± 1.79				
	8		76.40 ± 4.00			96.5 ± 5.0	

[a] Metubine iodide (dimethyl tubocurarine iodide, Lilly) is approximately 5 times as active as *d*-tubocurarine chloride in rats administered by vein.
[b] Metubine iodide (dimethyl tubocurarine iodide, Lilly) is approximately 4 times as active as *d*-tubocurarine chloride in the isolated gastrocnemius-sciatic nerve of frog.

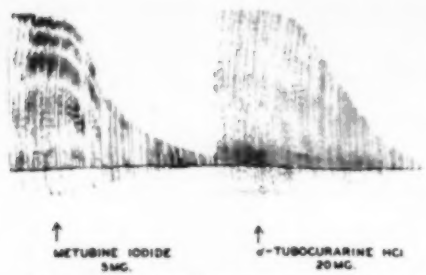

Fig. 2.—The isolated gastrocnemius nerve of a frog. 5 mg. of Metubine iodide caused the same inhibition of electrically induced contractions as 20 mg. of *d*-tubocurarine.

mately the same inhibition of electrically induced contractions as shown in Fig. 2 was 20 mg. for *d*-tubocurarine and 5 mg. for dimethyl ether *d*-tubocurarine; thus the methylated alkaloid is approximately four times as active as *d*-tubocurarine (Table III).

Isolated Phrenic Nerve-Diaphragm Method.—Doses of 0.2 mg. of *d*-tubocurarine in a 100-cc. bath produced an average inhibition of 27.5 ± 2.49% of the electrically induced muscle contractions as shown in Table IV and Fig. 3. The eight samples of the methylated derivative of 20.96 ± 0.69% to 33.64 ± 2.88% produced a mean of 25.95 ± 2.54% inhibition. Thus, the dimethyl ether alkaloid is approximately four times as active as *d*-tubocurarine.

Isolated Rectus Abdominus of Frogs.—As shown in Table IV and Fig. 4, *d*-tubocurarine in a dose of 0.05 mg. in the 10-cc. bath produced an average inhibition of 26.68 ± 1.32% of the acetylcholine-induced contractions. For the eight samples of the methylated alkaloid, the mean inhibition was 27.11 ± 2.88% with a dose of 0.06 mg.; thus the dimethyl ether derivative is 0.833 times as active as *d*-tubocurarine. Previously (1) we reported that if equimolar solutions were used, the methylated derivative was equal to *d*-tubocurarine in producing inhibition of acetylcholine-induced contractions.

It is interesting to note that in the frog's isolated gastrocnemius-sciatic preparation, the methylated alkaloid is approximately four times as active as *d*-tubocurarine, whereas in the rectus abdominus muscle of the same animal the methylated alkaloid is only about 80% as active.

DISCUSSION

This study was essentially concerned with comparison of the potency of dimethyl ether *d*-tubocurarine iodide with that of U. S. P. *d*-tubocurarine chloride by various methods. It appears obvious that the two intravenous methods, namely, the U. S. P. method and the single dose cross-over method utilizing rabbits, and the sloping screen method in rats subcutaneously, give rise to the same ratio of activity between *d*-tubocurarine and the methylated alkaloid.

The above methods have the advantage of using the whole animal, but they do not prove the usefulness of the preparations in medicine. Substances

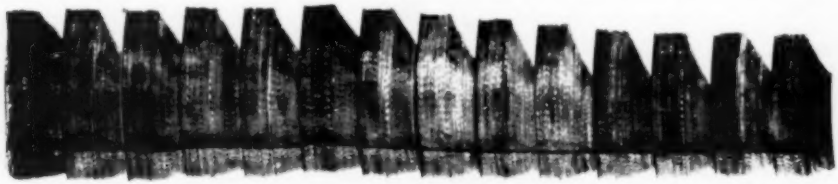

Fig. 3.—The isolated phrenic nerve-diaphragm of a rat. 200 μg. of *d*-tubocurarine produced the same inhibition of electrically induced contractions as 50 μg. of Metubine iodide.

TABLE IV.—COMPARATIVE ACTIVITY OF *d*-TUBOCURARINE CHLORIDE U. S. P. AND DIMETHYL ETHER *d*-TUBOCURARINE IODIDE

		Isolated Diaphragm-Phrenic Nerve Method, Rats			Isolated Rectus Abdominus Muscle Method, Frogs		
Drug	Sample	Dose, Mg.	Inhibition, %	Geometric Mean ± S. E., %[a]	Dose, Mg.	Inhibition, %	Geometric Mean ± S. E., %[b]
d-Tubocurarine	U. S. P.	0.200	.....	27.5 ± 2.49	0.05	.....	26.68 ± 1.32
Dimethyl ether *d*-tubocurarine iodide	1	0.050	31.16 ± 1.72	25.95 ± 2.54	0.06	26.68 ± 4.05	27.11 ± 2.88
	2		23.67 ± 4.05			27.29 ± 3.25	
	3		23.31 ± 1.76			27.51 ± 1.04	
	4		29.3 ± 3.70			26.42 ± 2.40	
	5		33.64 ± 2.88			26.68 ± 3.55	
	6		23.51 ± 2.23			26.44 ± 1.96	
	7		30.21 ± 2.12			28.03 ± 2.17	
	8		20.96 ± 0.69			27.05 ± 3.25	

[a] Metubine iodide (dimethyl tubocurarine iodide, Lilly) is approximately 4 times as active as *d*-tubocurarine chloride in rats.

[b] Metubine iodide (dimethyl tubocurarine iodide, Lilly) is approximately 0.833 times as active as *d*-tubocurarine chloride in frogs.

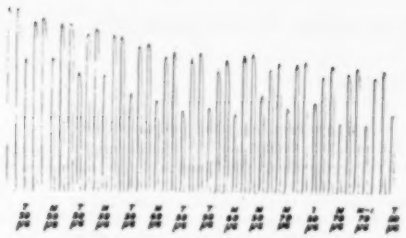

Fig. 4.—The isolated rectus abdominus muscle of frog. 60 μg. of Metubine iodide produced the same degree of inhibition of acetylcholine-induced contractions as 50 μg. of *d*-tubocurarine.

which cause head-drop or paralysis are not necessarily effective agents in anesthesiology. Myanesin is a good example. A more reliable method is the intact gastrocnemius-sciatic nerve preparation of the rat or cat. It is, however, only approximately quantitative and may not be a practical biological procedure. The isolated diaphragm-phrenic nerve preparation of the rat is perhaps the most accurate and practical method. Incidentally, it is now official in the British Pharmacopoeia.

Species differences present an important factor in any bioassay method. Wien (13) reported that on the isolated diaphragm-phrenic nerve of the rabbit, Flaxedil was equal to *d*-tubocurarine, whereas in the kitten it had one-fifth the activity of *d*-tubocurarine, and less than one-hundredth that of *d*-tubocurarine in the rat. As observed in this study, the mouse and the isolated rectus abdominus of the frog showed the methylated alkaloid to be equal in potency to that of *d*-tubocurarine when compared in equimolar solutions, but it was several times more active in the rabbit, in the rat, and in isolated phrenic nerve-diaphragm and gastrocnemius-sciatic experiments in the rat. Furthermore, in the frog's isolated gastrocnemius-sciatic preparation, the dimethyl ether derivative was four times as potent as *d*-tubocurarine, but it was only 80% as active as *d*-tubocurarine on the frog's isolated abdominus rectus muscle. This difference in results by two different preparations in the same animal (frog) must be attributed to the different mechanisms of action. The theory of acetylcholine-neuromuscular transmission has been universally adopted. Feldberg (14) in his discussion of the physiology of neuromuscular transmission and neuromuscular block stated that *d*-tubocurarine acts by reducing the depolarizing action of acetylcholine, so that the amounts usually released are insufficient to produce this required change at the end-plate. The gastrocnemius-sciatic method involves the action of curare alkaloids on the naturally occurring acetylcholine, while the rectus abdominus experiment measures their antagonistic action to acetylcholine which is constantly added. The dimethyl ether of *d*-tubocurarine must be less effective in the latter case.

CONCLUSIONS

1. Several biological methods have been used to determine the comparative potency of dimethyl ether *d*-tubocurarine iodide trihydrate and U. S. P. *d*-tubocurarine chloride pentahydrate.

2. In rabbits by the U. S. P. method and the single dose cross-over method, the methylated alkaloid was 9.02 ± 0.33 and 8.25 ± 0.50 times as active, respectively, as *d*-tubocurarine.

3. In rats the potency of the dimethyl ether derivative was 8.04 ± 0.33 times more potent than *d*-tubocurarine, but in mice it was only 0.803 ± 0.048 or one-tenth as active as in rats.

4. The methylated alkaloid was approximately five times more potent on the intact gastrocnemius-sciatic nerve of rats, approximately four times more active on the isolated gastrocnemius-sciatic nerve of frogs, and approximately four times more active on the isolated phrenic nerve-diaphragm of rats, than was *d*-tubocurarine.

5. On the isolated rectus abdominus of frogs, the methylated alkaloid was only approximately 0.833 times as active as *d*-tubocurarine, or was equal in potency with equimolar quantities of each alkaloid.

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Relative Action of Sunscreen Compounds*

By W. D. KUMLER†

A simple and rapid method of evaluating the relative screening action of sunscreen compounds is given. The optical density of a 0.1 per cent solution in a 0.1-mm. fused silica cell is measured at 3,080 Ångströms, the peak of the "Sunburn Curve." The optical density is divided by the per cent concentration, thus reducing all values to those given by a 1 per cent solution. These values are termed the sunscreen index (S. I.) of the compounds. Values of the sunscreen index are given for 45 compounds. An example is given showing how the sunscreen index may be used to calculate what per cent of any of the compounds is needed in a given thickness of film to screen out any given per cent of the light which produces sunburn.

SINCE a number of compounds are available for filtering out the ultraviolet rays which produce sunburn, a simple method of evaluating the relative effectiveness of such compounds is desirable. It was pointed out by Kumler and Daniels (1) that the long wave-length band of the erythema wave-length curve was not sufficient to decide where the maximum absorption of sunscreens should be, since the amount of sunburn produced depends not only on this curve but also on the amount of sunlight in the erythema region reaching the subject. The amount of this light reaching the earth depends on a number of factors, some of which are the time of day, the latitude, the amount of sunspot activity, the amount of ozone in the upper atmosphere, the amount of dust in the air, etc. The sunlight intensity wave-length curve used (1) was for normal sea level conditions and was similar to those reported by Moon (2). If the subject is at some higher altitude the curves of Götz (3) would apply. However, the amount of shift in the intensity wave length curve in going to an altitude of 10 kilometers is not very great and even at 15 kilometers there are no wave lengths of appreciable intensity below 2,900 Ångströms (4). Obviously that portion of the erythema curve below which there is no light from the sun is not pertinent to the sunburn problem. Therefore there is no need for considering any wave lengths below 2,900 Ångströms.

The amount of sunburn is dependent on the product of the intensity of light of a particular wave length reaching the skin times the relative effectiveness of that wave length in producing erythema. The ordinates of the erythema curve were multiplied by the ordinates of the sunlight energy wave-length curve, thus obtaining the "Sunburn Curve" (1). The peak at 3,080 Ångströms gives the wave length where maximum protection is needed. The base of the curve extends from approximately 2,900 to 3,260 Ångströms. This curve is sufficiently narrow so that if a compound has good absorption at its peak the probability is high that the compound will have adequate absorption to screen out effectively the wave lengths covered by the other portions of the curve. The relative screening power of different sunscreen compounds can thus be effectively compared by making one measurement of the optical density at 3,080 Ångströms in solutions of the proper concentration.

EXPERIMENTAL

The compounds measured in this investigation, with the exception of ethyl *p*-dimethylaminobenzoate, were supplied to us in 1% solution in propylene glycol by M. G. de Navarre. The solutions were measured with a Beckman ultraviolet spectrophotometer, using fused silica cells of 0.1-mm. path length. The more effective compounds could not be adequately evaluated at this concentration and path length because their densities were too high. Solutions of these compounds were then diluted tenfold, using 95% alcohol resulting in 0.1% solutions which brought the densities down to a value where the relative absorbing power of the various compounds for the rays producing sunburn could be compared.

The relative effectiveness of the compounds in stopping the sunburn producing rays is expressed in Table I as a number obtained by dividing the optical density of the solution at 3,080 Ångströms by the per cent concentration of the solution. The values, termed the "Sunscreen Index," then represent the density of a 1% solution of the compounds at a path length of 0.1 mm. The fact that isobutyl *p*-aminobenzoate has a higher value than the propyl compound appears anomalous and may be due to the relative purity of the compounds, which, with the exception of ethyl-*p*-dimethylaminobenzoate, were commercial products not purified further.

The optical density has been chosen to compare the different compounds, rather than the per cent transmission, because sunburn depends on the amount of light absorbed by the skin in the sunburn range, and the amount of light that is screened out is given directly by the optical density. On the other hand, the light screened out is a log function of the transmission.

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† Thanks are due to Mr. M. G. de Navarre who supplied the 1% solutions of forty-five compounds and to Dr. O. R. Wulf for several helpful discussions.

TABLE I

Compound	Sunscreen Index (S. I.) Optical Density at 3,080 Å. Conc., %
Ethyl *p*-dimethylaminobenzoate	14.8
Ethyl *p*-aminobenzoate	9.6
Isobutyl *p*-aminobenzoate	9.2
Propyl *p*-aminobenzoate	9.0
n-Butyl *p*-aminobenzoate	8.0
β-Methyl umbelliferone	7.7
p-Aminobenzoic acid	7.4
Dehydracetic acid	7.0
3-Carbethoxycoumarin	6.6
Benzilidine camphor	6.6
Heliotropine	6.5
Umbelliferone acetic acid	6.0
Salicylic acid	4.3
Sodium *p*-aminosalicylate	4.3
Methyl salicylate	4.0
Salicylamide	3.9
Methylenedisalicylic acid	3.0
3-Carboxycoumarin	3.0
Thiosalicylic acid	2.7
Brightener W/450	2.7
p-Hydroxyanthranilic acid	2.6
Sodium salicylate	2.4
Digalloyltrioleate	2.3
α-Resorcylic acid	2.2
Salicylaldehyde	2.2
p-Aminosalicylic acid	1.9
Dipropyleneglycol salicylate	1.9
Pyribenzamine	1.8
Pyrianisamine maleate	1.7
Sodium gentisate	1.7
Fluorescent white	1.6
Ethanolamide of gentisic acid	1.5
Ethyl gallate	1.4
Sodium sulfadiazine	1.2
Ethyl vanillate	1.1
Sodium sulfapyridine	0.95
Lauryl gallate	0.85
Totaquine	0.80
Barbituric acid	0.19
Chlorophyll	0.15
Amberlite IR-4-B	0.15
Salicyl alcohol	0.05
Anisic acid	0.01
Uvitex RBS	0.01
Uvitex RS	0.005

The numbers in Table I for the "Sunscreen Index" (S. I.) can then be used to compare the effective sunscreening power of any compound with any other compound on a weight basis. Thus propyl *p*-aminobenzoate is 9.0/3.0 = 3 times as effective, and the ethyl *p*-dimethylaminobenzoate is about 5 times as effective as 3-carboxycoumarin as far as the screening power of equal weights of the compounds are concerned. Now this does not mean that effective sunscreening preparations cannot be made from agents lower down in the table, because by using a higher concentration of an agent low in the list the same screening can be achieved as with a low concentration of some other agent above it. Thus a 3% solution of 3-carboxycoumarin would have as much protective action as a 1% solution of propyl *p*-aminobenzoate, considering only the relative absorbing characteristics of the compounds themselves.

Sometimes other factors enter into the effectiveness of the ultimate preparations, such as the thickness of the film that is formed on the skin when the preparation is applied, the decomposition of the compound in the presence of light, etc. If this film is twice as thick in one case as in another, the protective action of the one preparation would be double that of the other, although the two preparations might contain equal amounts of the same sunscreen compound. Factors of this type are not taken into account by the figures in Table I. This and perhaps some other factors can only be evaluated by actual tests on human subjects. However, one is frequently concerned not with these other factors but only with the relative effectiveness of various compounds on a weight basis in absorbing the rays that cause sunburn, and on that basis, the values of the sunscreen index given in Table I have significance.

The "Sunscreen Index" (S. I.) of the compound can be used to calculate the per cent of the compound that should be incorporated to screen out a certain percentage of the sunburn-producing rays under any given set of conditions. For example, the armed forces have set up specifications that a preparation shall not transmit more than 1% of the rays in the sunburn region through a film 0.001 inch thick. One can calculate what per cent of any one of the above compounds would have to be incorporated in a preparation to give this amount of screening. The figures in Table I are for a cell 0.1 mm. or $0.1/10 \times 2.5 = 0.004$ inch thick. A transmission of 1% corresponds to an optical density of 2. Therefore if x is the per cent of the compound that would have to be incorporated for 1% transmission under these conditions, then

$$x = \frac{2 \times 4}{\text{S. I.}} = \frac{8}{\text{S. I.}}$$

Thus for *n*-butyl *p*-aminobenzoate, x would be equal to $x = (8/8) = 1\%$ and for methyl salicylate it would have a value of $x = (8/4) = 2\%$. This signifies that a 1% solution of the former and a 2% solution of the latter when present in a film 0.001 inch thick would transmit 1% of the rays in the sunburn region. Since the specifications call for a transmission of less than 1%, the actual concentration used would have to be higher. Allowing for a safety factor of 100%, the concentration for *n*-butyl-*p*-aminobenzoate should be 2% and for methyl salicylate 4%.

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A Phytochemical Investigation of the Fruit of *Maclura pomifera* (Rafinesque) Schneider. I.*,†

By JOHN G. WAGNER‡ and LOYD E. HARRIS§

A phytochemical investigation of the fruit of *Maclura pomifera* (Rafinesque) Schneider has been undertaken. The collection and drying of the fresh fruit and the extraction of the dried fruit with various solvents is described. A preliminary report is given on the isolation of a new triterpene alcohol from the unsaponifiable matter of the petroleum ether extract of the dried fruit. Evidence is presented that D-glucose and D-fructose are present in the latex of the fresh fruit and in the cold absolute alcohol extract of the dried fruit. A pectic substance, which yielded mucic acid on oxidation with nitric acid, has been isolated from the one per cent aqueous hydrochloric acid extract. Evidence is presented that a pentosan, or some other hydrolyzable hemicellulose, is present in the five per cent aqueous sodium hydroxide extract.

MACLURA POMIFERA (Rafinesque) Schneider (1), commonly known as the Osage Orange, grows extensively throughout the southern, midwestern, and eastern states. The tree was described botanically by Rafinesque (2) and by Schneider (3) under the name *Ioxylon pomiferum*. The female trees bear a large fruit having an average diameter of $3^1/_2$ inches and an average weight of $1^1/_2$ pounds (4).

The phytochemical investigation of the fruit was undertaken as a result of reports that the whole fruit and some of its constituent parts exhibited certain pharmacological actions. A survey of the literature revealed that some phytochemical investigation of the fruit had been carried out but that further research might be of value. McHargue (4) reported that "the pulp and seed of the Osage Orange contain valuable feed, fertilizer, oil, and resin constituents." The isoflavone pigments, osajin and pomiferin, were isolated from the fruit and their structures proved by Wolfrom, *et al.* (5). Lupeol, a monohydric triterpene alcohol, was isolated from the fruit by Swift and Walter (6). Clopton and Roberts (7) stated that the water-soluble sugars present in the fruit consist mainly of a mixture of glucose, arabinose, and an unidentified disaccharide, but gave no experimental details to substantiate their report. Beal and Wenzel (8) established the nature of some of the constituent fatty acids in the oil which was obtained by extraction of the dried fruit with petroleum ether. In the present investigation, the dried fruit was extracted successively with a series of solvents. The extracts obtained and the latex of the fresh fruit were investigated individually.

EXPERIMENTAL

Collection of Fruit

The fruit used in this study was collected in October, and immediately after collection was placed in a refrigerated room.

Drying of Fruit

The fruit was washed, weighed, comminuted, and dried in an oven. The best method of comminution was to cut the fruit into thin slices by means of a mechanical slicer. The best method of drying was to dry the thinly sliced fruit to constant weight in an air-conditioned oven at a temperature of 80° (approximately twenty-four hours). The average loss in weight on drying the fresh fruit at 80° is approximately 82%. The dried fruit had a light reddish-brown color and an odor resembling Grape-nuts. The powdered, dried fruit was stored in completely filled, sealed jars to which were added a few drops of chloroform, which acted as a preservative.

Extraction of Dried Fruit

The powdered dried fruit was extracted successively with various solvents according to the procedure described by Rosenthaler (9). Using a Wester extractor, 200.0 Gm. of Sample II of the dried fruit was extracted with a series of solvents in the order given in Table I. For each organic solvent, the extraction was continued until no residue was obtained on evaporation of a sample from the extractor. The solvents were removed from extracts 1–5, inclusive, by distillation under reduced pressure at a temperature not above 38°.

The extracts were dried to constant weight in a vacuum desiccator over Drierite. The weights of the extracts are given in Table I.

Investigation of the Dry Extracts

Petroleum Ether Extract.—The 47.0 Gm. of petroleum ether extract (a brown oil) was heated under reflux with 2.4 L. of petroleum ether. After

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TABLE I.—EXTRACTION OF 200.0 GM. OF DRIED FRUIT (SAMPLE II)

Extract	Vol. Solvent, Ml.	Wt. of Dry Extract, Gm.	% Wt., Based on Dried Fruit	% Wt., Based on Fresh Fruit
Petroleum ether (b. p. 30–60°)	800.0	47.0	23.5	4.30
Diethyl ether	800.0	4.50	2.25	0.41
Chloroform	800.0	1.26	0.63	0.12
Cold absolute alcohol	800.0	31.5	15.7	2.89
Boiling 70% alcohol	800.0	34.0	17.0	3.11
Cold water	800.0			
Boiling water	400.0			
1% Cold, aqueous hydrochloric acid	1,000.0	45.7[a]	22.9[a]	4.19[a]
5% Aqueous sodium hydroxide	1,000.0			
1% Boiling, aqueous hydrochloric acid	800.0			
Residual marc		36.0	18.0	3.30

[a] Obtained by calculation.

cooling to room temperature the suspension was filtered; a fine yellow powder was retained on the filter paper. This yellow powder was composed principally of the pigments osajin and pomiferin, and was combined with the diethyl ether extract. The petroleum ether was distilled from the filtrate, the residual oil was saponified with alcoholic potassium hydroxide, and the unsaponifiable matter was extracted with ether. The separation of the constituents of the unsaponifiable matter constituted the major part of the investigation. The isolation of the triterpene alcohol, lupeol, from the unsaponifiable matter was reported by Swift and Walter (6). In the present investigation, lupeol, as well as a new triterpene alcohol isomeric with lupeol, was isolated from the unsaponifiable matter. The new triterpene alcohol was characterized as follows: alcohol, m. p. 166–168°, $[\alpha]_D^{23°}$ + 15.8° (EtOH, C = 1.044); acetate, m. p. 131–132° (corr., 132.5 to 133.5°), $[\alpha]_D^{25.5°}$ + 19.9° ($CHCl_3$, C = 4.399); benzoate, m. p. 124–125° (corr., 125.5 to 126.5°), $[\alpha]_D^{27.5°}$ + 43.7° ($CHCl_3$, C = 3.146). The name *lurenol* is suggested for this new triterpene alcohol. The characterization of lupeol and lurenol and their method of separation from the unsaponifiable matter will be the subject of a later paper.

Diethyl Ether Extract.—The combined diethyl ether extract and the yellow powder obtained from the petroleum ether extract (see above) were recrystallized from 60 ml. of xylene (activated charcoal); the yield of crude mixed pigments was 13.34 Gm., having a melting point of 177–180°. Fractionation of the crude mixture by the method of Wolfrom and Mahan (10) yielded 3.97 Gm. of pomiferin, m. p. 200.0 to 200.5° (corr., 204.0 to 204.5°), and 1.80 Gm. of osajin, m. p. 191.0 to 191.5° (corr., 195.0 to 195.5°). Wolfrom, *et al.*, reported a melting point of 200.5° (uncorr.) for pomiferin and a melting point of 189° (corr., 193°) for osajin.

Cold Absolute Alcohol.—This extract was a brown, viscous syrup, 97% of which dissolved in 100 ml. of water. The aqueous solution gave negative tests for alkaloids with Mayer's reagent, 5% phosphomolybdic acid in 10% nitric acid, and saturated picric acid solution. The aqueous solution gave a positive Molisch test and a positive Selivanoff test. An osazone was formed from the aqueous solution by the procedure of Shriner and Fuson (11), a known sugar, D-glucose, being used for comparison. The osazone was shown to be phenyl-D-glucosazone by means of melting point, mixed melting point, and crystal structure.

Using an apparatus and procedure for ascending paper chromatography on large-size filter sheets similar to that described by Wolfson, Cohn, and Devaney (12), paper chromatograms were developed, using the aqueous solution prepared from the absolute alcohol extract. The solvent used to develop the chromatograms was the organic phase of an equilibrated mixture of benzyl alcohol (3 parts), water (3 parts), and acetic acid (1 part) described by Jermyn and Isherwood (13). The chromatograms, after development for about twenty-two hours in a sealed glass tank saturated with water vapor, were dried at 80°, sprayed with a mixture of equal parts of 0.1 *N* $AgNO_3$ and 5 *N* NH_4OH, and then heated for a few minutes at 80°. Known sugars were used for comparison. The paper chromatograms indicated that D-glucose and D-fructose were present in the water-soluble portion of the absolute alcohol extract.

Boiling 70 Per Cent Alcohol Extract.—This extract was a dark brownish-red, very viscous syrup. Titrations with Benedict's quantitative sugar reagent showed that the dried extract contained 32% reducing sugars calculated as glucose. This value was not increased by acid hydrolysis.

One Per Cent Cold Aqueous Hydrochloric Acid.—Slight precipitation occurred on storage of the extract in the refrigerator. After filtration, there was insufficient residue on the filter to carry out any identification tests. The filtrate measured 850 ml., and a 750-ml. portion was concentrated by distillation under reduced pressure to about two-thirds of its original volume. To the concentrated solution was added an equal volume of 95% ethanol. The gelatinous precipitate which formed was removed by filtration, washed with 95% ethanol, and dried in a desiccator. The yield was 55.7 Gm. of a jelly-like material which was oxidized with nitric acid by the procedure described by Beilstein (14), and the product was recrystallized from water. The yield was 1.97 Gm. of long, colorless, needle-shaped crystals having a melting point of 99.5 to 100.0° (corr., 100.1 to 100.6°), unchanged on admixture with a hydrated mucic acid of the same melting point prepared from lactose. When some of the colorless needles were washed on a filter paper with a small amount of hot water, followed immediately by absolute alcohol and then

ether, 0.15 Gm. of a fine white, microcrystalline powder, having a melting point of 211.0 to 212.0° (corr., 215.4 to 216.4°) was obtained. One of the reported (11) melting points of mucic acid is 213° with decomposition. The experimental evidence indicated that the jelly-like material was a pectic substance and that the needle-shaped crystals, m. p. 99.5 to 100.0°, were a hydrated form of mucic acid. These crystals, when treated successively with hot water, alcohol, and ether, yielded a non-hydrated form of mucic acid.

Five Per Cent Aqueous Sodium Hydroxide Extract.—This extract was neutralized with hydrochloric acid and the volume was reduced to 100 ml. by evaporation. To the concentrated solution was added 50 ml. of 95% ethanol. The dark brown precipitate which formed was removed by filtration on a 1/4-in. bed of Celite 535 filter aid. The residue, mixed with Celite, gave a positive Millon's test and a positive Biuret test for protein. The residue gave a negative test with Benedict's solution for reducing saccharides, but after hydrolysis with hydrochloric acid and subsequent neutralization of the hydrolysate with sodium bicarbonate, a Benedict's test was positive. The remainder of the residue was tested for pentosans by the A. O. A. C. method (15) involving distillation with 12% hydrochloric acid. The distillate contained furfural, as shown by the phloroglucinide obtained when phloroglucinol was added to some of the distillate, and by the red color which developed when some of the distillate was treated with aniline and acetic acid. The results indicated the presence of a pentosan, or some other hydrolyzable hemicellulose, in the 5% aqueous sodium hydroxide extract.

The Latex of the Fresh Fruit

The fruit of *Maclura pomifera* contains a milky, viscous latex which has been reported to contain certain enzymes, carbohydrates, fats, and albumins (16). In this study, a yield of 528.5 Gm. of strained latex was expressed from 4,997 Gm. of fresh fruit by means of a hand press. Refrigeration of the latex at 0° for three days in a stoppered flask caused separation into a jelly-like mass surrounded by an almost clear, amber-colored liquid. Part of the filtered liquid (34 Gm.) yielded 1.2 Gm. of a crude osazone by the procedure of Shriner and Fuson (11). One-half of the crude osazone, on recrystallization, yielded 0.3 Gm. of phenyl-D-glucosazone, m. p. 205.0° (corr. 209.5°), unchanged on admixture with an authentic specimen of phenyl-D-glucosazone prepared from D-glucose. A microscopic examination of the osazone crystals indicated they were identical to the known osazone and to photomicrographs of phenyl-D-glucosazone. The other half of the crude osazone yielded 0.03 Gm. of phenyl-D-glucosotriazole, m. p. 194–195°, prepared by the method of Hann and Hudson (17).

The amber-colored liquid, which separated from the latex on standing, gave a positive Selivanoff test for a ketose and gave the specific test for fructose described by Dehn, Jackson, and Ballard (18). A negative test for mannose (18) was obtained and attempts to prepare mannose phenylhydrazone and mannose 2,4-dinitrophenylhydrazone from this liquid were unsuccessful.

The development of large circular paper chromatograms (12), using the solvent mixture (*n*-butanol 40%, ethanol 10%, and water 50%) described by Partridge (19), indicated that the liquid which separated from the latex contained D-glucose and D-fructose.

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A Phytochemical Investigation of the Fruit of *Maclura pomifera* (Rafinesque) Schneider*,†

II. Isolation of a New Triterpene Alcohol

By JOHN G. WAGNER‡ and LOYD E. HARRIS§

Acetylation of the unsaponifiable matter of the petroleum ether extract of the dried fruit yielded a crystalline acetate fraction which was separated by fractional crystallization into lupenyl acetate and a new triterpenyl acetate for which the authors have suggested the name *lurenyl acetate*. Saponification of lurenyl acetate yielded the crystalline alcohol lurenol. When lurenol was benzoylated, both lurenyl benzoate and lupenyl benzoate were separated from the benzoylation mixture. When lurenol was acetylated, both lurenyl acetate and lupenyl acetate were separated from the acetylation mixture. These isomerizations and the results of analytical data indicate that lurenol is a monohydric triterpenyl alcohol, isomeric with lupeol, and has the molecular formula $C_{30}H_{49}OH$.

IN A PREVIOUS PUBLICATION (1) the authors made a preliminary report on the isolation of a new triterpene alcohol from the fruit of *Maclura pomifera*, commonly known as the Osage Orange. The isolation and characterization of this new alcohol are reported in detail herein.

Lupeol, a monohydric triterpene alcohol, was isolated from the unsaponifiable matter of the petroleum ether extract of the dried fruit of *Maclura pomifera* by Swift and Walter (2). The structure of lupeol has not been entirely elucidated (3), but Ruzicka and Rosenkrantz (4) and Ames and Jones (5) have formulated tentative structures.

Amyrins, mostly β-amyrin (an isomer of lupeol), were reported to be present in a concentration of 0.91 per cent in the dried fruit of the Osage Orange by Clopton and Roberts (6); however, they gave no experimental details and reported no physical constants. No evidence has been found in this investigation that either α- or β-amyrin is present in the fruit of *Maclura pomifera*.

In the present investigation, a procedure different from that used by Swift and Walter (2) has been applied to the separation of the constituents of the unsaponifiable matter.

EXPERIMENTAL

Isolation of the Unsaponifiable Matter.—Extraction of 2,347 Gm. of dried fruit (1) with low-boiling petroleum ether (4,430 ml.) in a Soxhlet apparatus for eighteen and one-half hours yielded 385 Gm. of oil (16.4% of the dried fruit). Saponification of the oil with alcoholic potassium hydroxide and extraction of the unsaponifiable matter with ethylene chloride (Eastman Kodak Company) and ether yielded 43.15 Gm. (Sample I) of unsaponifiable matter (1.86% of the dried fruit).

Extraction of 3,000 Gm. of dried fruit with low-boiling petroleum ether (4,500 ml.) in a Soxhlet apparatus for twenty-eight hours yielded 351 Gm. of oil (11.7% of the dried fruit). Saponification of the oil with 0.5 *N* alcoholic potassium hydroxide and extraction of the unsaponifiable matter with ether yielded 83.5 Gm. (Sample II) of unsaponifiable matter (2.78% of the dried fruit).

Direct Recrystallization of the Unsaponifiable Matter.—Thirty grams of unsaponifiable matter (Sample I) was recrystallized twice from acetone and once from 85% ethanol. Two fractions of crystalline material were obtained: one fraction, m. p. 195.0 to 197.0°, weighed 2.00 Gm.; the other fraction, m. p. 151.5 to 154.5°, weighed 1.30 Gm No further crystalline material with a satisfactory melting point range could be isolated, since a yellowish-brown, resinous substance precipitated on cooling the solutions. No pure lupeol, or any other substance with a constant melting point, was isolated from the crystalline fractions. A quantitative recovery of the remainder of the unsaponifiable matter was obtained by combining all the acetone and alcoholic mother liquors, distilling the solvents, and drying the residue to constant weight at 80°. This residue weighed 26.70 Gm. and is herein designated the "unsaponifiable residue."

Acetylation of the Unsaponifiable Matter.—A mixture of 23.70 Gm. of "unsaponifiable residue," 287 ml. of acetic anhydride, and 23.7 Gm. of anhydrous sodium acetate was heated at 135° ± 5° for three hours. The acetylation mixture was cooled to room temperature, and the crude crystalline acetates were removed by filtration, washed with water until free of acid, and then dried at 100° ± 5° for three hours. The yield of mixed acetate with a melting-point range of 129–170° was 18.65 Gm. The wash waters and the filtrate of the reaction mixture were poured into a suspension of ice and water and stirred mechanically for one

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hour. The semisolid, brown, resinous masses which precipitated were extracted with ether. The brown ethereal solution was washed with carbonate and then with water. Evaporation of the ether yielded a yellowish-brown, resinous residue which, when dried at 100°, weighed 8.0 Gm.

By the same procedure, 83.5 Gm. of unsaponifiable matter (Sample II) yielded 63.5 Gm. of crude, crystalline acetate and 23.5 Gm. of the resinous residue described above.

Fractional Crystallization of the Crude Acetates.—Recrystallization of the 18.65 Gm. of crude acetates from approximately absolute ethanol (500 ml.) yielded three fractions: (*a*) 10.39 Gm. with a melting-point range of 156–177°; (*b*) 5.72 Gm. with a melting-point range of 125–129°; and (*c*) 1.97 Gm. of yellowish-brown, resinous residue. The fractions (*a*) and (*b*) were submitted to an extensive series of recrystallizations from absolute alcohol, alcohol-acetone, and alcohol-acetone-water mixtures. The less soluble, higher melting-point fractions ultimately yielded 4.76 Gm. of lupenyl acetate, m. p. 216.0 to 216.5° (corr., 222.3 to 222.8°), $[\alpha]^{28.5°}_{D}$ +42.3° ($CHCl_3$, C = 2.009). The more soluble, lower melting-point fractions ultimately yielded 6.91 Gm. of a new triterpenyl acetate (lurenyl acetate), m. p. 131.0–132.0° (corr., 132.5 to 133.5°), $[\alpha]^{28.5°}_{D}$ +19.9° ($CHCl_3$, C = 4.399).

Recrystallization of the 63.5 Gm. of crude acetates from approximately absolute alcohol (1,250 ml.) yielded a fraction weighing 29.67 Gm., with a melting-point range of 177–187°, and a more soluble fraction. The more soluble fraction was subjected to a series of recrystallizations from ethanol, ethanol-water, and acetone-water. The yield was 6.24 Gm. of lurenyl acetate, m. p. 131.0 to 132.0° (corr., 132.5 to 133.5°), $[\alpha]^{30°}_{D}$ +20.1° ($CHCl_3$, C = 3.310), in the form of microscopic, colorless rods. The melting point of the lurenyl acetate was unchanged on recrystallization from acetone-water (three different ratios) and from absolute alcohol.

Anal.—Calcd. for $C_{30}H_{49}OCOCH_3$: C, 81.98; H, 11.18. Found:[1] C, 81.57; H, 10.76; C, 81.63; H, 10.87.

The saponification equivalent determined by the method of Shriner and Fuson (7) was found to be 470.9; the saponification equivalent of $C_{30}H_{49}OCOCH_3$ is 468.7.

Lupeol and Lupenyl Benzoate.—The saponification of 3.00 Gm. of lupenyl acetate was carried out in 0.5 *N* alcoholic potassium hydroxide at reflux temperature. After one recrystallization from ethanol-water, the lupeol weighed 2.58 Gm. The melting point of the lupeol was 214.5 to 215.0°; $[\alpha]^{28.5°}_{D}$ +27.0° ($CHCl_3$, C = 4.476).

One gram of lupeol was benzoylated by dissolving it in 30 ml. of anhydrous pyridine, adding 1 ml. of benzoyl chloride, and heating the solution at 115–130° for three hours. Three recrystallizations of the crude benzoate from benzene-ethanol mixture yielded 0.51 Gm. of lupenyl benzoate in the form of large, flat, brittle, colorless plates, having a m. p. of 261.0 to 263.0° (corr., 269.2 to 271.2°); $[\alpha]^{28.5°}_{D}$ +59.2° ($CHCl_3$, C = 1.000). The benzoate recovered from the mother liquors and that recovered from the determination of the specific rotation were combined (0.39 Gm.) and recrystallized from acetone. The yield was 0.23 Gm. of lupenyl benzoate in the form of long, colorless needles having a m. p. of 264.0 to 266.0° (corr., 271.7 to 273.7°); and 0.14 Gm., having a m. p. of 263.9 to 265.0° (corr., 271.8 to 273.8°).

Lurenol.—The saponification of 1.65 Gm. of lurenyl acetate was carried out by refluxing it with 0.5 *N* alcoholic potassium hydroxide on a steam bath for one and one-half hours in an atmosphere of nitrogen. After distillation of about half of the ethanol under reduced pressure (capillary carrying nitrogen), the lurenol was extracted with ether. The ether extract was decolorized with activated charcoal and the ether was removed by distillation under reduced pressure (capillary carrying nitrogen). Crystallization of the crude lurenol from 80% ethanol-water yielded 0.813 Gm. of lurenol in the form of double- and single-pointed, colorless needles and rosettes of needles. The alcohol melted at 154.0 to 156.0° (corr., 156.5 to 158.5°) to a turbid liquid which cleared at 161° (corr., 163.8°); $[\alpha]^{33°}_{D}$ +15.8° (EtOH, C = 1.044). Recrystallization of 200 mg. of the alcohol from ethanol-water yielded 177 mg. of colorless needles, m. p. 166.0–168.0° (corr., 168.8 to 170.8°). The melting point was unchanged after heating the lurenol in a vacuum oven at 100° ± 2° for three hours. Similarly, 100 mg. of the alcohol on recrystallization from 80% ethanol-water yielded 46 mg. of colorless needles, m. p. 166.0 to 168.0°, which melting point was unchanged on one further recrystallization from the same solvent.

Anal.—Calcd. for $C_{30}H_{49}OH$: C, 84.45; H, 11.81. Found: C, 84.73; H, 11.78.

Lurenyl Benzoate and Isomerization.—The saponification of 3.10 Gm. of lurenyl acetate yielded 2.47 Gm. of crude lurenol, obtained partly as an oil and partly in crystalline form. Benzoylation of the crude lurenol with benzoyl chloride and anhydrous pyridine yielded 3.19 Gm. of crude benzoate. The crude benzoate on recrystallization from acetone-ethanol yielded high and low melting-point fractions. The high melting-point fraction, after recrystallization twice from acetone, yielded 0.30 Gm. of lupenyl benzoate, m. p. 261.0 to 263.0° (corr., 269.2 to 271.2°). On admixture with lupenyl benzoate of the same melting point (described above), the melting point was unchanged. The low melting-point fraction was subjected to a series of recrystallizations from acetone-ethanol, acetone-methanol, acetone-methanol-water, and ethanol-water. The yield was 1.22 Gm. of a benzoate, assumed to be lurenyl benzoate, m. p. 124.0 to 125.0° (corr., 125.5 to 126.5°); $[\alpha]^{27.5°}_{D}$ +43.7° ($CHCl_3$, C = 3.146). The melting point of the lurenyl benzoate was unchanged on recrystallization from various solvent mixtures.

Anal.—Calcd. for $C_{30}H_{49}OCOC_6H_5$: C, 83.72; H, 10.25. Found: C, 83.98; H, 9.49; C, 84.06; H, 9.27.

The saponification equivalent determined by the method of Shriner and Fuson (7) was found to be 514.7; the saponification equivalent of $C_{30}H_{49}OCOC_6H_5$ is 530.8.

In another experiment, starting with some of the lurenol (m. p. 154.0 to 156.0°), similar results were obtained.

[1] All microanalyses reported in this paper were made by the Clark Microanalytical Laboratory, Urbana, Ill.

Acetylation of Lurenol and Evidence of an Isomerization.—Acetylation of 85 mg. of lurenol, m. p. 154.0 to 156.0°, by a reaction with acetic anhydride and anhydrous sodium acetate, yielded 59 mg. of crude acetates. Recrystallization of the crude acetates from acetone-water in a centrifuge tube yielded two fractions: the first fraction of crystals weighed 20 mg. and melted at 211–212°; the second fraction of crystals weighed 18 mg. and melted at 126–127°. Recrystallization of the first fraction from absolute ethanol yielded 9 mg. of lupenyl acetate, m. p. 216.0 to 216.5°. This melting point was unchanged on admixture of a sample with a sample of lupenyl acetate of the same melting point (described above). Recrystallization of the second fraction twice from acetone-water yielded 6 mg. of lurenyl acetate, m. p. 131.0 to 132.0°. This melting point was unchanged on admixture with a sample of lurenyl acetate of the same melting point (described above).

Attempted Isomerization of Lurenyl Acetate under Acetylation Conditions.—A solution of 0.50 Gm. of lurenyl acetate in 15 ml. of acetic anhydride was heated at 137–140° for seven hours. The solution was colorless for about one hour, but some resinification occurred and the solution gradually changed to a yellow color. The hot solution was treated as an acetylation mixture and was worked up in the usual way. Recrystallizations of the acetate recovered from ethanol-water and acetone-water yielded 0.38 Gm. of lurenyl acetate, m. p. 131.0 to 132.0°; the melting point was unchanged on admixture with the starting material. The recovery was 76% and would have been higher if resinification during the prolonged heating period had not occurred. No trace of lupenyl acetate, which could have been detected by its much higher melting point, was found.

DISCUSSION

When lurenyl acetate is saponified, the crystalline alcohol, lurenol, is obtained. Reacetylation of lurenol yields both lupenyl acetate and lurenyl acetate. Benzoylation of lurenol yields lupenyl benzoate and a low melting-point benzoate which is assumed to be lurenyl benzoate. Hence it is evident that somewhere in each of these sequences of reactions an isomerization occurs. The isomerization could occur: (*a*) during the saponification of lurenyl acetate, (*b*) during the formation of the acetate or benzoate from lurenol, or (*c*) after the acetate or benzoate is formed. Possibly (*a*) would require that the lurenol isolated be a mixture containing lupeol. Certain evidence to be presented indicates that the isomerization does not occur in this step. The inability to convert any lurenyl acetate to lupenyl acetate under acetylation conditions appears to exclude possibility (*c*). Although not conclusive, it appears that the isomerization occurs during the formation of the acetate or benzoate from lurenol. Many isomerizations, under various conditions, have been reported in the triterpene field (8–10).

SUMMARY

One kilogram of dried fruit yielded 18.39 Gm. of unsaponifiable matter, which after acetylation yielded 14.47 Gm. of crude triterpenyl acetates. Fractional recrystallization of the acetate mixture yielded 5.36 Gm. of lurenyl acetate, m. p. 131.0 to 132.0° (corr., 132.5 to 133.5°) and 3.69 Gm. of lupenyl acetate, m. p. 216.0 to 216.5° (corr., 222.3 to 222.8°). Saponification of lurenyl acetate yielded the crystalline alcohol, lurenol. Reacetylation or benzoylation of lurenol yielded both the corresponding lupeol and lurenol derivatives.

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A Phytochemical Investigation of the Fruit of *Maclura pomifera* (Rafinesque) Schneider*,†

III. Color Tests, Paper Chromatography, and Infrared Spectra of Lurenol, Lupeol, and Their Derivatives

By JOHN G. WAGNER‡ and LOYD E. HARRIS§

The Liebermann-Burchard and Salkowski color tests were applied to lurenol, lupeol, and their acetates. The infrared spectra of these compounds have been recorded. Paper chromatography was applied to lurenol, lupeol, their acetates and benzoates.

In a previous publication (1) the authors reported the isolation of two triterpene alcohols, lurenol and lupeol, from the fruit of *Maclura pomifera*, commonly known as the Osage Orange. Lupeol was originally isolated from the fruit by Swift and Walter (2) but lurenol had not formerly been reported to be present in the fruit.

In the course of the present research it was desirable to find methods to differentiate the triterpenols and their derivatives in very small quantities. The Liebermann-Burchard reaction (3) has been applied to a wide variety of compounds, including some triterpenes (2, 4). The authors found this reaction and the Salkowski test (3) useful in differentiating lurenol, lupeol, and their acetates. The Liebermann-Burchard test was also applied to the fresh and dried latex of the fruit of *Maclura pomifera*. The sequences of colors obtained indicated the presence of lurenol or lurenyl acetate or both in the fresh latex of the fruit, and the sequence of colors obtained when the test was applied to the dried latex was different from that reported formerly (2). A paper chromatographic method was developed which may be useful in application to other triterpenes.

The infrared spectra of lurenol, lurenyl acetate, lupeol, and lupenyl acetates are recorded. The spectra of the alcohols are very similar but differ in some details, and the same is true of the spectra of the acetates. Interpretation of the spectra on an empirical basis (5, 6) indicated that lurenol contains a hydroxyl group and one or more double bonds.

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† Abstract of a dissertation presented to the Graduate School of the Ohio State University by John G. Wagner in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This study was supported in part by a fellowship grant by the American Foundation for Pharmaceutical Education.

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EXPERIMENTAL

Color Tests.—When the Liebermann-Burchard test (3) was applied to 1–2 mg. of either lurenol or lurenyl acetate, a green-colored solution which exhibited a strong green fluorescence was immediately obtained. The color changes were: green → yellowish-green → yellowish-brown → red globules in a yellowish-brown liquid. Lupeol and lupenyl acetate, under the same conditions, gave only a slowly developing pink color, which deepened within a few minutes. Lupenyl acetate gave a less intense color than lupeol. There was no green fluorescence. Although Swift and Walter (2) reported that lupeol gave a red color with the Liebermann-Burchard reagents, the authors found that a red color could be produced with these reagents only by adding an excess of sulfuric acid.

When the Salkowski test (3) was applied to lurenyl acetate, a violet-to-purple color developed within one-half hour in the chloroform layer. Under the same conditions, lurenol, lupeol, and lupenyl acetate gave only a colorless chloroform layer.

When a few drops of the fresh latex of the fruit of *Maclura pomifera* was shaken with 2 ml. of chloroform for five minutes and the suspension was filtered, the filtrate gave a Liebermann-Burchard test (3) which was the same as that described for lurenol and lurenyl acetate. This indicated the presence of lurenol or lurenyl acetate or both in the fresh latex of the fruit.

Some of the white, milky latex of the fruit was dried at 100° to a brown, brittle solid. This solid was shaken with chloroform and the suspension was filtered. When the Lierbermann-Burchard test (3) was applied to the filtrate, a green-colored solution which exhibited a strong green fluorescence was obtained immediately. The green color changed to yellowish-green, then to yellowish-brown. Ten minutes later the solution was dark red and contained red globules. This was a different result from that reported by Swift and Walter (2) for the fruit; they mentioned only the final red color.

Infrared Spectra.—The infrared spectra of lurenol (Fig. 1), lupeol (Fig. 2), lurenyl acetate (Fig. 3), and lupenyl acetate (Fig. 4) were obtained in the Spectrographic Laboratory of the Chemistry Department of the Ohio State University with the help of Dr. C. L. Wilson. The instrument used was the Baird Associates infrared recording spectrophotometer with sodium chloride prism. The concentration used in each case was 10% w/v. The solvent was anhydrous chloroform. The brief analysis (Table I) of the spectra indicates the groups responsible for some of the absorption bands; assignments were made on an empirical basis (5, 6).

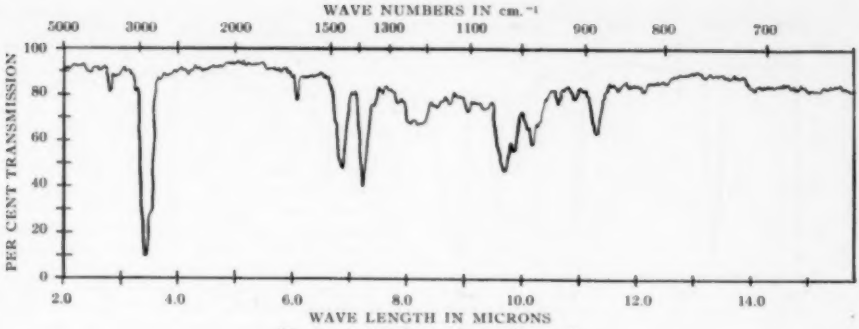

Fig. 1.—Infrared spectrum of lurenol.

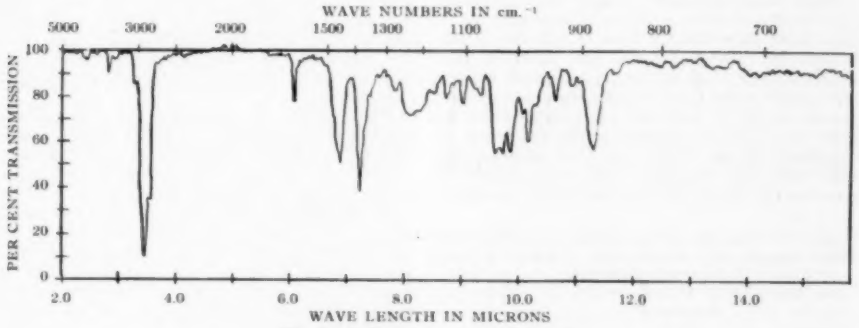

Fig. 2.—Infrared spectrum of lupeol.

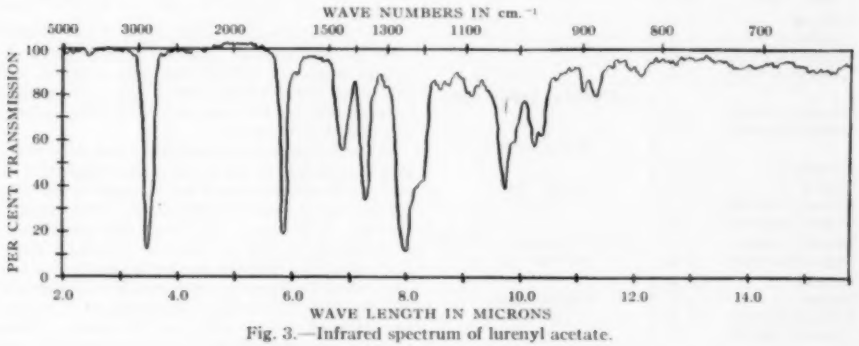

Fig. 3.—Infrared spectrum of lurenyl acetate.

The following points are noteworthy. The spectra of lurenol and lupeol are very similar, but differ in some details, and the same is true of the spectra of lurenyl acetate and lupenyl acetate. The small size (low intensity) of the hydroxyl band of lurenol and lupeol indicates a relatively small amount of hydroxyl in the molecule since the molecules are very large. There is apparently no carbonyl group in the lurenol molecule since there is no band at 5.8 μ (1,724 cm^{-1}) which is characteristic of the carbonyl in such molecules as acetone (5).

The spectra of lurenyl acetate and lupenyl acetate have a marked resemblance to the spectrum of cholesteryl acetate. However, even though cholesteryl acetate contains a carbon-to-carbon double bond, its infrared spectrum shows little or no absorption band on the long wave-length side of 6.0 μ which is characteristic of this grouping (5). The spectra of lurenol, lurenyl acetate, lupeol, and lupenyl acetate all exhibit an absorption band in this region, indicative of the presence of a carbon-to-carbon double bond in each of these molecules.

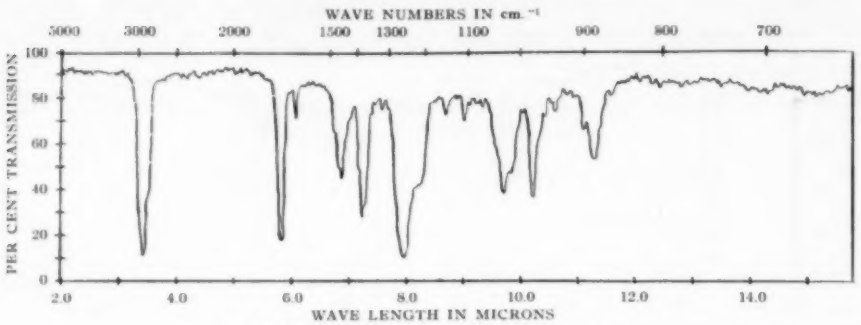

Fig. 4.—Infrared spectrum of lupenyl acetate.

Paper Chromatography.—Paper chromatograms were developed on large sheets of Whatman No. 1 filter paper shaped in the form of cylinders as described by Wolfson, Cohn, and Devaney (7). Approximately 10–20 μg. of the compound desired was applied to the paper in a circular area, approximately 5 mm. in diameter, by spotting 0.001 to 0.002 ml. of a 1% solution of the compound in chloroform at the origin line, then allowing the chloroform to evaporate. Individual solutions were spotted alternately at the origin line, the spots being approximately 6 cm. apart. The paper cylinders had a circumference of 39.5 cm. and a total height of 29.5 cm.

TABLE I.—EMPIRICAL ASSIGNMENT OF GROUPS RESPONSIBLE FOR ABSORPTION BANDS IN THE INFRARED SPECTRA

Compound	Absorption Band Peak, μ	Assigned Grouping
Lurenol	2.80	Hydroxyl (—OH)
Lupeol	2.80	
Lurenol	3.41	C—H
Lupeol	3.41	
Lurenyl acetate	3.43	
Lupenyl acetate	3.41	
Lurenyl acetate	5.82	Carbonyl of Ester Group (R—C(=O)—OR′)
Lupenyl acetate	5.80	
Lurenol	6.05	Double Bond (C=C)
Lupeol	6.07	
Lurenyl acetate	6.10	
Lupenyl acetate	6.05	
Lurenyl acetate	8.00	Ether Linkage of Ester (C—O—C)
Lupenyl acetate	7.95	
Lurenol	11.3	Double Bond (C=C)
Lupeol	11.3	
Lurenyl acetate	11.25	
Lupenyl acetate	11.3	

The paper cylinder was then placed upright in a crystallizing dish. A beaker (400 ml.) of water was placed in the center of the dish. This assembly was lowered into a large, cylindrical glass tank having a diameter of 29 cm. and a height of 30 cm. A ground-glass plate, sealed with silicone stopcock lubricant, was fitted to the top of the glass tank. The ground-glass plate had an eccentric hole 2.3 cm. in diameter and 5 cm. from one edge. This hole served as a means to add solvents to the tank and to insert a thermometer into the tank while the chromatograms were being developed. The tank was leveled by means of spirit levels placed at right angles.

The apparatus was allowed to stand for one and one-half to twelve hours to saturate the filter paper with water. At the end of this period, the thermometer was removed and a solvent mixture was introduced into the crystallizing dish at the base of the paper cylinder by means of a long glass tube and a funnel. Preliminary experiments disclosed that the best solvent mixture for the compounds studied was a mixture of 100 ml. of 95% ethanol, 40 ml. of methanol, and 35 ml. of water. After the solvent mixture had been introduced, the tank was quickly sealed by reinserting the thermometer. The solvent was allowed to ascend the paper cylinder for a determined period of time (five and one-half to seven hours).

The paper cylinder was removed from the tank after the development period, the location of the solvent boundary was marked with a pencil, and the paper cylinder was dried in an air-circulating oven at 105° for five minutes. The dried cylinder was then cut into strips.

By preliminary investigation, it was determined that the location on the paper chromatograms of all compounds studied, except lupeol, could be found by the method of Bush (8) who applied the method to steroids. The paper strips were first dipped into a saturated solution of iodine in petroleum ether. Each strip was allowed to dry in front of a fan. The violet background color quickly disappeared but the locations of the compounds were marked by yellowish iodine spots on the paper. These yellow spots faded rapidly, hence they were quickly outlined with a pencil. The strips were then dipped quickly into an aqueous solution containing 1% arrowroot starch and 2% potassium iodide. The yellow spots became blue against a pale-blue background. After the strips were dried at room temperature or at 105°, the blue spots changed to violet or reddish brown while the background became a light reddish brown. A permanent record of the chromatograms was thus obtained.

The R_F value, $\frac{\text{distance traveled by the spot}}{\text{distance traveled by the solvent}}$,

for each compound on each chromatogram was determined. The R_F value of lurenyl acetate was 0.26 ± 0.04. In all experiments under the same conditions the lupenyl acetate spots remained at the origin, hence the R_F value of lupenyl acetate was zero. The R_F value of lurenyl benzoate was 0.21 ± 0.07. The wide range was due to "tailing" of the spots. In all experiments under the same conditions the lupenyl benzoate spots remained at the origin, hence the R_F value of lupenyl benzoate was zero. The R_F value of lurenol was 0.31 ± 0.02. On many of the strips, no colored spot corresponding to lupeol was observed. When a few of the strips were held in front of a light immediately after the treatment with the starch solution, some faint-blue streaks were observed which extended away from the origin line where lupeol was spotted. These streaks indicated some "tailing" of the lupeol spot. No such streaks were observed extending from the origin line where lurenol had been spotted. Although not conclusive evidence, this indicated that the lurenol samples contained no lupeol as an impurity. No spot at the origin line ever appeared where lurenyl acetate or lurenyl benzoate had been spotted. This indicated that the lurenol derivatives contained no corresponding lupeol derivative as an impurity. No spot ever appeared above the origin line at the point where either lupenyl acetate or lupenyl benzoate had been spotted. This indicated that the lupeol derivatives contained no corresponding lurenol derivative as an impurity. This is substantiated by a typical experiment in which a mixture of lurenyl acetate and lupenyl acetate, with a melting point range of 187–191°, was separated on a paper chromatogram by the same procedure. Spots corresponding to both lurenyl acetate and lupenyl acetate were obtained.

Sample photographs of some of the chromatograms are shown in Figs. 5–8 inclusive. The purpose for which the chromatogram shown in Fig. 6 was prepared was to show that different samples of lurenyl acetate were homogeneous and that recrystallization did not change the R_F value of lurenyl acetate under the given set of conditions. The sample of lurenyl acetate (1.65 Gm.) was isolated after acetylation of the 83.5 Gm. of unsaponifiable matter (1). The sample of lurenyl acetate (3.11 Gm.) was isolated after acetylation of the 23.70 Gm. of "unsaponifiable residue" (1). The sample (0.25 Gm.) of lurenyl acetate was obtained by recrystallizing 0.39 Gm. of the 3.11-Gm.

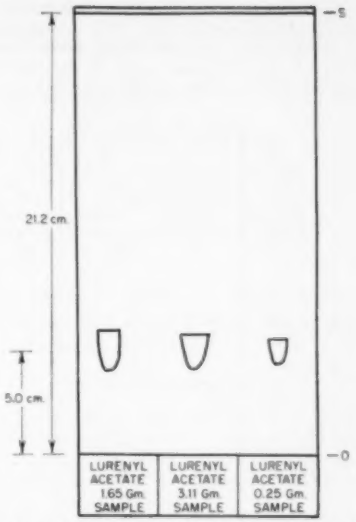

Fig. 6.—Paper chromatogram comparing three different samples of lurenyl acetate.

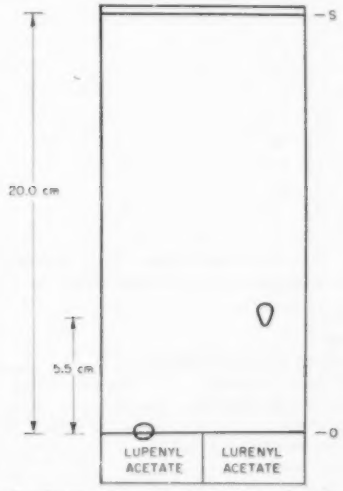

Fig. 5.—Paper chromatogram comparing lupenyl acetate with lurenyl acetate.

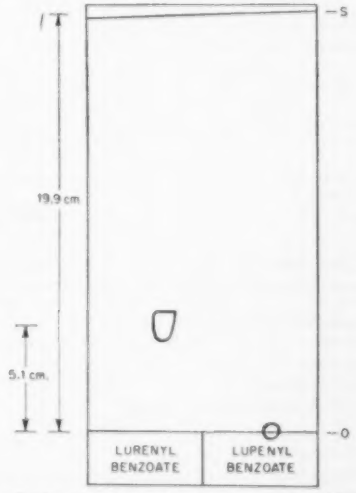

Fig. 7.—Paper chromatogram comparing lupenyl benzoate with lurenyl benzoate.

sample three times, the first time from methanol-water (10:1), the second time from acetone-water (6:1), and the third time from acetone-water (5.5:1). A mixed melting point of the 0.25-Gm. sample and the 3.11-Gm. sample of lurenyl acetate

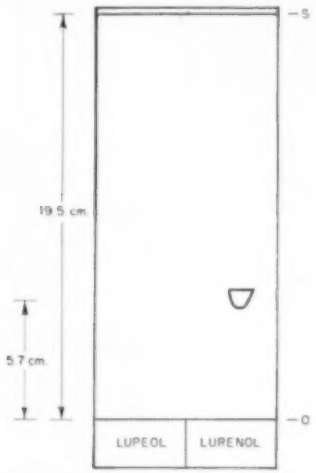

Fig. 8.—Paper chromatogram comparing lupeol with lurenol.
O—Origin line where compounds spotted.
S—Solvent boundary.

was the same as the melting point of each sample, namely 131.0 to 132.0° (corr., 132.5° to 133.5°). After a seven-hour development period, each of the lurenyl acetate spots had moved up the paper a distance of 5.0 cm., whereas the solvent boundary had moved 21.2 cm. Each of the lurenyl acetate samples, therefore, had the same R_F value, namely, $\frac{5.0}{21.2}$ or 0.24.

SUMMARY

1. Lurenol and lurenyl acetate give a sequence of colors in the Liebermann-Burchard test different from that of lupeol and lupenyl acetate.

2. Color tests indicate that lurenol or lurenyl acetate or both are present are the fresh and dried latex of the fruit of *Maclura pomifera*.

3. The infrared spectra of lurenol, lurenyl acetate, lupeol, and lupenyl acetate are recorded.

4. Comparisons are made between lurenol and lupeol, lurenyl acetate and lupenyl acetate, and lurenyl benzoate and lupenyl benzoate by paper chromatography. This method detects very small quantities of the compounds, provides a means of separation of small quantities of the compounds, and possibly provides a criterion to show that the samples of each isolated are homogeneous.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal, and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Neopyrithiamine
Novirudim
Pyrimidine
n-Tridecylamine
5,9,10-Trimethylbenzanthracene
1,11-Undecanedioic acid
Ceryl alcohol
Butyrylcholine
Secretin
Riboflavine-5-phosphoric acid
Pitessine
Oxyindol
8-Methoxy-2-methylquinoline
Ethyl-*d*-pantothenate
Diethylarsenic bromide
Camphorene
Selachyl alcohol
Muconic acid
Glucamine
Coproporphyrin III

A Statistical Study of Some Disintegrating and Binding Agents in Certain Compressed Tablets*

By ELVIN A. HOLSTIUS† and H. GEORGE DeKAY‡

The purpose of this work was to attempt to show some causes for the variation in disintegrating times of various types of tablets. Three types of tablets, representing the soluble tablet, the insoluble tablet, and the complex tablet, were used in this investigation. Three different types of tablets were then used in preparing tablet granulations, employing different binding or massing agents. The data obtained were treated statistically by a three-way analysis using equations designed by the Statistical Laboratory of Purdue University.

MEDICATION as compressed tablets has long been recognized as one of the most convenient methods of oral administration in unit dosage form. Such prominence has imposed several requirements, foremost of which is rapid disintegration.

Several workers (1–7) have proposed methods of hastening disintegration, mainly by incorporating some agent which has rapid swelling qualities. Milne (8), and Granberg and Benton (9), suggest the possibility of methyl cellulose or bentonite.

Of all the disintegration studies conducted and reported, none has presented statistical data to substantiate the conclusion reached. It must be assumed that the results are of a basic, comparative nature, indicating superficial differences and thereby disregarding any interactions which are present in all such problems.

As most compressed tablets are composed of more than one ingredient and require several steps in manufacture, it is possible that these factors greatly affect disintegration rates for any given tablet. Factors influencing disintegration include the nature of the active constituent, disintegrant, binder, size and weight, hardness, and the method used to determine disintegration rate.

On the basis of these variabilities, which must be considered collectively, it appears that the present methods of statistical evaluation would tend to evaluate justly the variables involved, reduce the human tendencies to a norm, and permit definite conclusions to be reached.

The statistical study applied was an analysis of variance designed for the original data by the Statistical Laboratory of Purdue University. The F-test applied to the data is based upon an estimation of the variances and affords an insight as to whether or not the results are significant in terms of probability.

EXPERIMENTAL

To maintain the same variables in the several batches of tablets investigated, definite requirements for each batch and the method of measuring disintegration rate had to be established and maintained as closely as possible.

Three types of tablets were prepared; namely, sulfathiazole as representative of an organic constituent, sodium bicarbonate as representative of an inorganic constituent, and compound aspirin as representative of a complex tablet.

Binders employed as representative of those commonly used included gelatin-acacia solution, sucrose solution, and starch paste.

Disintegrants included arrowroot, corn, potato, sweet potato, rice, sorghum, tapioca, and wheat starches, bentonite, and sodium alginate.

Hardness was maintained within relatively close tolerances by means of a Monsanto Hardness Tester. The same size and weight tablet was prepared for all batches by using a small Stokes Model E single-punch machine with a $^{3}/_{8}$-inch die and standard, concave, stainless steel punches.

Disintegration rate was determined by means of the modified DeKay, Evanson, and Sperandio method (10, 11). The time required for the last granule to pass through an 8-mesh screen was accepted as the end point of disintegration.

All tablets were prepared according to the following formula:

Active constituent(s)	324.6 mg.
Filler	38.9 mg.
Disintegrant	38.9 mg.
Binder	*q. s.*
Lubricant	3.9 mg.
Total	406.3 mg.

The active constituent (except for aspirin which was obtained in granular form from Dow Chemical Company of Midland, Mich.), the filler (lactose), and the disintegrant were passed through a 60-mesh screen and mixed well. The powders were moistened with a solution of the binder employed and then were granulated through a 20-mesh screen and allowed to dry. The dried granules were passed through a 20-mesh screen, lubricated with magnesium stearate (previously screened through an 80-mesh sieve), and compressed. Hardness was main-

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‡ Grateful acknowledgment is made to Mr. C. Springer of the Mathematics Department, Purdue University, for assistance with the statistical procedures and evaluations.

TABLE I.—ORIGINAL AVERAGE DISINTEGRATION TIME IN SECONDS FOR ALL VARIABLES[a]

Active Constituent	Binding Agent[b]	Disintegrant: Arrowroot Starch	Corn Starch	Potato Starch	Sweet Potato Starch	Rice Starch	Sorghum Starch	Tapioca Starch	Wheat Starch	Bentonite	Totals
Sulfathiazole	A	923	374	1,078	1,261	835	623	875	872	1,655	8,496
	B	1,123	501	822	661	610	579	740	413	903	6,352
	C	139	341	225	81	79	403	169	212	1,786	3,435
Sodium bicarbonate	A	564	499	281	446	727	614	950	644	1,920	6,645
	B	287	345	528	656	826	533	831	451	1,150	5,607
	C	417	250	294	264	410	442	773	376	855	4,081
Aspirin compound	A	68	142	240	165	169	161	171	103	582	1,801
	B	573	369	417	207	241	428	409	419	1,040	4,103
	C	22	25	25	34	42	34	39	29	54	304
Totals		4,116	2,846	3,910	3,775	3,939	3,817	4,957	3,519	9,945	40,824

[a] All disintegration times have been rounded to the next higher second, to facilitate ease of reading and comparison, in Table I and all subsequent tables.
[b] A = gelatin-acacia solution; B = sucrose solution; C = starch paste.

tained at approximately 4.0 Kg./cm². Disintegration times were then observed on 10 groups of 10 tablets each, using an electric mini-second timer.

Table I is a composite of the original disintegration times in seconds observed for the three active constituents, the three binding agents, and the nine disintegrating agents employed in this study.

Assuming that the times observed were a result of interaction effects, it was deemed advisable to utilize the analysis of variance (12–15). The results of the equations made it possible to determine, by means of the F-ratio, an estimation of the interaction effects, and on this basis to ascertain the probable actions of combinations of main effects under investigation.

The mean disintegration times were obtained by dividing the total of all individual observations by the total number of observations made. Tables II–V indicate the results obtained.

TABLE II.—MEAN DISINTEGRATION TIME FOR THE THREE ACTIVE CONSTITUENTS

Active Constituent	Time, Sec.
Sulfathiazole	675
Sodium bicarbonate	591
Aspirin compound	230

TABLE III.—MEAN DISINTEGRATION TIME FOR THE THREE BINDING AGENTS

Binding Agent Solution	Time, Sec.
Gelatin-acacia solution	613
Sucrose solution	595
Starch paste	287

TABLE IV.—MEAN DISINTEGRATION TIME FOR THE DISINTEGRATING AGENTS

Disintegrating Agent	Time, Sec.
Arrowroot starch	457
Corn starch	316
Potato starch	434
Sweet potato starch	420
Rice starch	438
Sorghum starch	424
Tapioca starch	551
Wheat starch	383
Bentonite	1,062

The interaction of the active constituents and the binding agents was found to be significant by means of the F-ratio. This indicates that the rate of disintegration is determined in part by the proper combinations of the active constituent and the binding agent.

TABLE V.—MEAN DISINTEGRATION TIME FOR THE ACTIVE CONSTITUENTS AND THE BINDING AGENTS

Binding Agent Solution	Time, Sec.: Sulfathiazole	Sodium Bicarbonate	Aspirin Compound
Gelatin-acacia solution	944	695	200
Sucrose solution	706	623	456
Starch paste	374	453	34

The interaction effects of the active constituent and the disintegrating agents were found to be significant, as shown in Table VI.

TABLE VI.—MEAN DISINTEGRATION TIME FOR THE ACTIVE CONSTITUENTS AND THE DISINTEGRATING AGENTS

Disintegrating Agents	Time, Sec.: Sulfathiazole	Sodium Bicarbonate	Aspirin Compound
Arrowroot starch	728	423	230
Corn starch	405	365	179
Potato starch	708	368	227
Sweet potato starch	668	455	136
Rice starch	508	654	151
Sorghum starch	535	530	208
Tapioca starch	595	851	207
Wheat starch	476	491	184
Bentonite	1,448	1,179	559

Realizing that Table VI compares only the disintegrating agents with the active constituents, we cannot definitely conclude that one disintegrant is of any greater value than any other, for these two components are not the only conditions governing disintegration time. Therefore, the interaction effects of the binding agents and the disintegrating agents were compared, as shown in Table VII.

By means of the F-ratio it was found that the interaction effects of the disintegrating agents and

TABLE VII.—MEAN DISINTEGRATION TIME FOR THE BINDING AGENTS AND THE DISINTEGRATING AGENTS

Disintegrating Agents	Time, Sec. Gelatin-Acacia Solution	Sucrose Solution	Starch Paste
Arrowroot starch	518	661	193
Corn starch	338	405	206
Potato starch	533	589	181
Sweet potato starch	624	508	126
Rice starch	577	559	177
Sorghum starch	466	513	293
Tapioca starch	665	660	327
Wheat starch	540	428	183
Bentonite	1,256	1,031	898

the binding agents were significant. Comparisons for degrees of significance were made according to the method devised by Brownlee (16). The residual mean square represents the amount of variability that cannot be attributed to any of the main effects or interactions, and must therefore be considered as a measure of the inherent experimental error.

In the three-way analysis (Table VIII), it was found that the main effects, i.e., the active constituent, the binder, and the disintegrating agents, did not exert any significant effect; yet there was an effect present when the three were in combination. This led to the opinion that the rate of disintegration was not due to any influence upon the part of the main constituents singularly, but that, when in certain combinations, their significance was notable. Therefore it was deemed advisable to analyze the problem by means of a two-way method. This eliminated one of the components of the interactions, and by comparing the two remaining effects against the interaction of the new analysis it was possible to determine the importance of these components upon the rate of disintegration.

The two-way analysis consisted of designing the table and calculations for each of the active constituents, and thus the effects of the binding agents and the disintegrating agents were determined separately and in combination as the interaction. By breaking the analysis down into its two components, it was possible to withdraw the pertinent data which was hidden in the three-way analysis.

By means of the F-ratio, it was found that the binding agents, regardless of the disintegrating agents, were definitely significant when employed in compressed tablets of sulfathiazole, and that the disintegrating agents were also significant. The F-ratio showed that the degree of significance was very high. This indicates that, with sulfathiazole, the rates of disintegration observed were dependent upon the relationship which exists between the disintegrating agents and the binding agents (Tables IX–XI).

TABLE IX.—MEAN DISINTEGRATION TIME FOR THE BINDING AGENTS IN SULFATHIAZOLE

Binding Agent	Time, Sec.
Gelatin-acacia solution	944
Sucrose solution	706
Starch paste	374

TABLE X.—MEAN DISINTEGRATION TIME FOR THE DISINTEGRATING AGENTS IN SULFATHIAZOLE

Disintegrating Agent	Time, Sec.
Arrowroot starch	728
Corn starch	405
Potato starch	708
Sweet potato starch	668
Rice starch	508
Sorghum starch	535
Tapioca starch	595
Wheat starch	476
Bentonite	1,448

TABLE XI.—MEAN DISINTEGRATION TIME FOR THE INTERACTION OF THE DISINTEGRATING AND BINDING AGENTS IN SULFATHIAZOLE

Disintegrating Agents	Gelatin-Acacia Solution	Sucrose Solution	Starch Paste
Arrowroot starch	923	1,123	139
Corn starch	374	501	341
Potato starch	1,078	822	226
Sweet potato starch	1,261	661	81
Rice starch	835	611	80
Sorghum starch	623	580	403
Tapioca starch	875	740	170
Wheat starch	872	413	212
Bentonite	1,655	903	1,786

TABLE VIII.—SUMMATION OF THREE-WAY ANALYSIS

Source of Variation[a]	Sum of Squares	Degrees Freedom	Mean Sum of Squares	F-Ratio	F 0.05	F 0.01
A	16,682,337.8	2	8,341,168.9	1.27[b]	5.14	10.92
B	4,664,193.0	2	2,332,096.5	<1.00[b]	...	...
C	21,432,788.0	8	2,679,098.5	1.15[b]	2.30	3.26
A × B	20,988,146.8	4	5,247,036.7	40.08[c]	2.67	3.97
A × C	22,830,858.3	16	1,426,928.6	10.90[c]	1.97	2.62
B × C	16,490,200.9	16	1,030,637.6	7.87[c]	1.97	2.62
A × B × C	4,189,139.0	32	130,910.6	7.90[d]		
Residual	11,986,111.2	729	16,441.9			
Total	119,263,775.0	809				

[a] A = active constituent; B = binding agents; C = disintegrating agents.

[b] These main effect F-ratios were computed by dividing the main effect mean square by a mean square which is a composite of the mean squares of the interactions involving this main effect.

[c] These F-ratios were computed by dividing the mean square by the mean square of the interaction active constituent × binding agent × disintegrating agent.

[d] This F-ratio was obtained by dividing the mean square for the interaction active constituent × binding agent × disintegrating agent by the mean square for the residual.

The F-ratio calculated for the effects of the binding agents in sodium bicarbonate tablets indicated that they were definitely significant and that it is highly probable that the binding agent is the most

TABLE XII.—MEAN DISINTEGRATION TIME FOR THE BINDING AGENTS IN SODIUM BICARBONATE

Binding Agent	Time, Sec.
Gelatin-acacia solution	695
Sucrose solution	623
Starch paste	453

important contributing factor to the rates of disintegration observed in this study. The F-ratio obtained for the disintegrating agents for sodium bicarbonate indicates that they are definitely signi-

TABLE XIII.—MEAN DISINTEGRATION TIME FOR THE DISINTEGRATING AGENTS IN SODIUM BICARBONATE

Disintegrating Agent	Time, Sec.
Arrowroot starch	423
Corn starch	365
Potato starch	368
Sweet potato starch	455
Rice starch	654
Sorghum starch	530
Tapioca starch	851
Wheat starch	491
Bentonite	1,179

ficant. By means of the F-ratio it was found that there is a high degree of significance for this interaction effect (Tables XII–XIV).

TABLE XIV.—MEAN DISINTEGRATION TIME FOR THE INTERACTION OF THE DISINTEGRATING AND BINDING AGENTS IN SODIUM BICARBONATE

Disintegrating Agents	Gelatin-Acacia Solution	Sucrose Solution	Starch Paste
Arrowroot starch	564	287	417
Corn starch	499	345	250
Potato starch	281	528	294
Sweet potato starch	446	656	264
Rice starch	727	820	410
Sorghum starch	614	533	442
Tapioca starch	950	831	773
Wheat starch	644	451	376
Bentonite	1,530	1,150	855

The F-ratio obtained for the binding agents in aspirin compound was significant. This indicates that the binding agents are probably more important

TABLE XV.—MEAN DISINTEGRATION TIME FOR THE BINDING AGENTS IN ASPIRIN COMPOUND

Binding Agent	Time, Sec.
Gelatin-acacia solution	200
Sucrose solution	456
Starch paste	34

to the disintegration times observed for this tablet than would be apparent by means of only comparative measurements. The F-ratio obtained for the

TABLE XVI.—MEAN DISINTEGRATION TIME FOR THE DISINTEGRATING AGENTS IN ASPIRIN COMPOUND

Disintegrating Agent	Time, Sec.
Arrowroot starch	221
Corn starch	179
Potato starch	227
Sweet potato starch	136
Rice starch	151
Sorghum starch	208
Tapioca starch	207
Wheat starch	184
Bentonite	559

disintegrating agents was found not to be significant; in any case where the F-ratio might be significant, it would be so in a very small percentage of instances (Tables XV–XVII).

TABLE XVII.—MEAN DISINTEGRATION TIME FOR THE INTERACTION OF THE DISINTEGRATING AND BINDING AGENTS IN ASPIRIN COMPOUND

Disintegrating Agent	Gelatin-Acacia Solution	Sucrose Solution	Starch Paste
Arrowroot starch	68	573	22
Corn starch	142	369	25
Potato starch	240	417	25
Sweet potato starch	165	207	34
Rice starch	169	241	42
Sorghum starch	161	428	34
Tapioca starch	171	410	39
Wheat starch	103	419	30
Bentonite	582	1,040	54

Comparisons for degrees of significances were made according to the method devised by Brownlee (16) (Tables XVIII–XX).

SUMMARY AND CONCLUSIONS

1. A statistical study of the analysis of variance of disintegration times observed was made with results presented in tabular form.

2. The disintegration times observed, and the statistical study made on these observations, indicate that there is no singular effect of the active constituent, binding agent, or disintegrating agent in the tablet which is the sole criteria in determining disintegration rate.

3. Rate of disintegration for a tablet is probably due to the interaction effects of the three main effects.

4. The statistical study indicates there is no universal disintegrating agent which would provide the same rate of disintegration in all tablets.

TABLE XVIII.—SUMMATION OF TWO-WAY ANALYSIS OF SULFATHIAZOLE

Source of Variation[a]	Sum of Squares	Degrees Freedom	Mean Sum of Squares	F-Ratio
A	14,707,456.1	2	7,353,728.1	7.85[b]
B	23,027,621.9	8	2,878,452.7	3.07[b]
A × B	14,978,388.5	16	936,149.3	62.97[c]
Residual	3,627,787.1	243	14,929.7	
Total	56,341,253.6	269	209,447.0	

[a] A = binding agents; B = disintegrating agents.
[b] These F-ratios were obtained by dividing the mean squares by the mean square of the interaction effect.
[c] This F-ratio was obtained by dividing the mean square of the interaction effect by the mean square for the residual.

TABLE XIX.—SUMMATION OF TWO-WAY ANALYSIS OF SODIUM BICARBONATE

Source of Variation[a]	Sum of Squares	Degrees Freedom	Mean Sum of Squares	F-Ratio
A	2,771,558.0	2	1,385,779.0	7.35[b]
B	17,362,495.5	8	2,170,311.9	11.51[b]
A × B	3,016,028.6	16	188,501.8	13.17[c]
Residual	3,476,220.6	243	14,305.4	
Total	26,626,302.7	269	98,982.5	

[a] A = binding agents; B = disintegrating agents.
[b] These F-ratios were obtained by dividing the mean squares by the mean square of the interaction effect.
[c] This F-ratio was obtained by dividing the mean square of the interaction effect by the mean square for the residual.

TABLE XX.—SUMMATION OF TWO-WAY ANALYSIS OF ASPIRIN COMPOUND

Source of Variation[a]	Sum of Squares	Degrees Freedom	Mean Sum of Squares	F-Ratio
A	8,139,674.4	2	4,069,837.2	8.85[b]
B	3,873,528.9	8	484,191.1	1.05[b]
A × B	2,718,591.4	16	169,911.9	1.00[c]
Residual	116,347,733.4	243	478,797.3	
Total	131,079,528.1	269	487,284.5	

[a] A = binding agents; B = disintegrating agents.
[b] Since the interaction, when tested against the residual, was not significant, the main effect F-ratios were determined by dividing their respective mean squares by a new estimate of the residual mean square formed from the interaction and the originally calculated residual (16).
[c] This F-ratio was obtained by dividing the mean square for the interaction effect by the mean square for the residual.

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Extraction of Prodigiosin from Blood Plasma and Body Tissues and Its Measurement by Spectrophotometric Analysis*,†

By GEORGE V. TAPLIN, JAMES S. GREVIOR, CLAYTON H. DOUGLAS, ARTHUR DUNN, CAMILLE FINNEGAN, and MARY LOUISE LaNIER

Satisfactory methods have been developed to extract prodigiosin from samples of blood plasma and various body tissues. They depend on the preferential solubility of the dye in petroleum ether or in acid chloroform over that in blood plasma or in tissue fluids. The concentration of prodigiosin in these extracts may be measured accurately by spectrophotometric analysis, using the appropriate wave lengths of the absorption band of the dye in the solvents. Amounts as small as 0.1 micrograms per milliliter may be detected in a reproducible manner.

FOR THE PAST three years, an extensive study has been made to clarify the mechanisms involved in the removal of insoluble particles from the respiratory tract following exposure to various types of dusts, in normal versus irradiated animals. Information regarding the fate of inhaled foreign material could be valuable in the management of atomic bomb casualties, as well as helpful in the understanding and evaluation of dust hazards in industry and in allergic respiratory disorders.

Prodigiosin is a tripyrolmethane dye which is produced from cultures of *Serratia Marcesens* (1–6). It was selected as a tracer substance because it may be readily pulverized to form an extremely fine water-insoluble dust, and because it has a distinct red color in dilutions up to one part per million. Also, colloidal aqueous suspensions of the dye may be prepared by wet-grinding the crystals with physiological amounts of dextrose and ascorbic acid. Moreover, the colloidal preparation is tolerated by rabbits when given intravenously in single doses not exceeding 1–2 mg./Kg. It is distributed rapidly within the body tissues in a pattern characteristic of other colloidal suspensions (7–9) and is not excreted in the urine. Furthermore, rabbits tolerate inhalation exposures to prodigiosin dust, and its distribution in the pulmonary tree may be observed grossly or detected microscopically in frozen sections.

The purpose of this paper is to describe the extraction procedures which have been found satisfactory, along with a spectrophotometric method of analysis, by which it is possible to determine the prodigiosin content in specimens of body fluids and tissues.

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EXPERIMENTAL

Extraction Methods and Technique of Prodigiosin Assay in Blood Plasma or Body Tissues

General Principles.—Prodigiosin[1] may be extracted from blood plasma and from most body tissues by the use of two solvents, namely, acid chloroform for tissues and alkaline petroleum ether for plasma. Inseparable emulsions are formed if chloroform is used to extract the dye from blood plasma. However, when plasma is alkalinized and extracted with a 1:1 mixture of petroleum ether and 95% ethyl alcohol, good separation is accomplished. The concentration of prodigiosin may be determined in either of these solvents by using the Beckman Model DU spectrophotometer, because the dye has peak absorption bands at 533 mμ in petroleum and 537 mμ in acid chloroform.

Reagents.—(*a*) Acid alcohol: 95% ethyl alcohol mixed with 3% glacial acetic acid by volume.

(*b*) Acid chloroform: Reagent grade chloroform mixed with 1% acid alcohol by volume.

(*c*) 5% sodium hydroxide.

(*d*) A 1:1 mixture of petroleum ether and 95% ethyl alcohol.

Extraction Procedures

Blood Plasma.—(*a*) Five cubic centimeters or more of oxalated blood is centrifuged at 3,000 r. p. m. for forty-five minutes.

(*b*) Two cubic centimeters of plasma is alkalinized with 1 cc. of 5% NaOH.

(*c*) Eight cubic centimeters of the petroleum ether-alcohol solvent is added and the mixture is shaken vigorously for two minutes.

(*d*) The test tube containing the mixture is stoppered and centrifuged at 2,000 r. p. m. for thirty minutes.

(*e*) The supernatant ether is transferred to a Corex cell and is acidified with 0.03 cc. of glacial acetic acid to convert the prodigiosin to its acid form (red color).

Body Tissues.—(*a*) Aliquot specimens of large organs or entire small organs are minced and a measured amount of acid chloroform is added. The mixture is ground in a Waring blendor for three minutes. A ratio of 1 Gm. of tissue to 5 cc.

[1] The crystalline prodigiosin base used in this study was prepared and provided by Dr. Arthur Lack of the Long Beach Veterans Administration Hospital, Long Beach, Calif.

TABLE I.—PRODIGIOSIN RECOVERY FROM RABBIT LIVER IN SINGLE AND DOUBLE EXTRACTIONS WITH ACID CHLOROFORM

Prodigiosin Added to Sample, μg./Gm.	Number of 5-Gm. Aliquots[a]	First Extraction		Second Extraction[b]	
		Prodigiosin Recovered, μg./Gm.	Mean Per Cent Recovered[c]	Prodigiosin Recovered μg./Gm.	Mean Per Cent Recovered
4.2	9	3.4	82.5 ± 7.3	0.6	14.2 ± 7.6
12.0	9	8.9	74.5 ± 8.6	2.6	21.6 ± 8.6
20.0	6	15.6	78.0 ± 5.5	3.6	18.0 ± 6.6

[a] Each aliquot was extracted with 25 cc. of acid chloroform.
[b] Total recovery of double extractions at the three concentrations of prodigiosin was approximately 96%.
[c] Standard deviations calculated by S. D. = $\sqrt{\frac{\Sigma n^2}{n-1}}$

of chloroform is used. The blending procedure should be limited to two to three minutes to avoid overheating and evaporation of the chloroform.

(*b*) The mixture is transferred to a large test tube and the chloroform containing the dye is separated by filtration through a Whatman #5 paper.

Spectrophotometric Analysis

Two standard curves showing the relationship between per cent transmission and prodigiosin concentration are prepared, one for tissue samples (acid chloroform solvent) and a second for plasma extracts (petroleum ether-ethyl alcohol solvent) (see Fig. 1). The data for plotting the curves are obtained by preparing a series of known concentrations of prodigiosin in each of the two solvent mixtures and by measuring the per cent transmission at the appropriate wave lengths in the absorption band of the dye: namely, 533 mμ for prodigiosin in petroleum ether and 537 mμ for chloroform. A slit width of 0.015 mm. is used in all determinations.

The prodigiosin content in tissue specimens is calculated by the following formula and by use of the chloroform curves shown in Fig. 1. Prodigiosin in μg/Gm. =

$$\frac{\text{Prodigiosin in } \mu\text{g/cc. solvent}^{2} \times \text{vol. solvent in cc.}}{\text{Wet weight of tissue samples in grams}}$$

The amount of prodigiosin in plasma samples is determined by measuring the per cent transmission in the extract and by converting this value to prodigiosin concentration by use of the standard curve of prodigiosin in petroleum ether (Fig. 1) and by the same formula used for tissue specimens.

Results Showing Biological Applications of the Method

The results obtained from single and double extractions of prodigiosin from aliquots of liver tissues are presented in Table I. The second extraction increases the per cent recovery. However, for semiquantitative analysis, a single extraction may be employed, and a correction factor may be used, if desired, to account for that fraction of prodigiosin retained in the tissue after a single extraction procedure. The efficiency of the method of determining plasma prodigiosin concentrations is presented in Table II. Measured amounts of the colloidal dye were added to normal plasma, and aliquot specimens were extracted and assayed separately. For comparative biological studies, a single extraction process gives sufficiently accurate and reproducible results.

TABLE II.—PRODIGIOSIN RECOVERY FROM RABBIT PLASMA[a]

Known Concentrations of Prodigiosin, μg./Ml. Plasma	Number of Aliquots	Prodigiosin Recovered, μg./Ml. Plasma	Recovery, %
1.25	9	1.15	92.4 ± 8.3
4.6	8	4.24	91.7 ± 6.8
48.0	10	43.8	92.9 ± 5.6

[a] Known amounts of prodigiosin were added to normal rabbit plasma. Extractions were made using 2-cc. aliquots of plasma and 8-cc. volumes of petroleum ether-alcohol solvent.

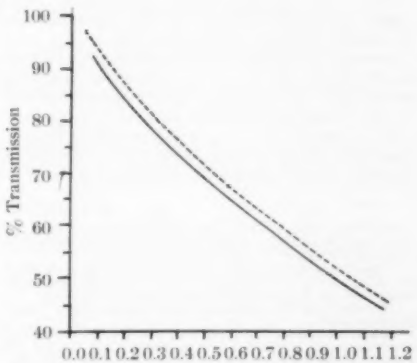

Fig. 1.—Prodigiosin concentration vs. per cent transmission. – – – prodigiosin in chloroform; ——prodigiosin in petroleum ether.

[2] Value for prodigiosin in μg/cc. chloroform is obtained by converting the value determined for per cent transmission into prodigiosin concentration (Fig. 1).

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Note

A Note on the Use of the Anderson-Craver Heart Perfusion Apparatus*,†

By JAMES L. LEITCH and THOMAS J. HALEY

WHILE investigating the effects of four antiheparin dyes on the isolated mammalian heart [Leitch and Haley, (1)], certain modifications in the use of the Anderson-Craver apparatus (2) were developed and are reported below.

EXPERIMENTAL

Silicone-coating of Apparatus.—Although the entire apparatus need not be completely coated with silicone, the flowmeter for measuring coronary flow and the air pump for returning the perfusate to the elevated reservoir should be treated. For this purpose, Desicote[1] [Gilbert, (3)] is the most satisfactory preparation.

Distilled Water for Perfusate.—An organic contaminant made the laboratory distilled water unsuitable for the preparation of the perfusate recommended by Chenoweth and Koelle (4). A suitable water is prepared by treatment of the laboratory distilled water with 0.5 Gm. of Darco carbon G-60[2] per liter and, after a minimum contact period of fifteen minutes, the complete removal of the carbon by filtration. No information is available, however, on the applicability of the above procedure to the distilled water of other laboratories.

Coronary Flowmeter Modifications.—To reduce the discharge volume of the flowmeter described by Anderson and Cameron (5), the space below the bottom of the float is filled by a siliconed, cylindrical piece of inert plastic, having a central hole slightly larger than the glass stem of the float. By use of this plastic insert, the volume per discharge may be reduced to any value between 4 ml. and the maximum volume of the flowmeter, depending on the over-all dimensions of the insert.

Injection of Dose.—When dyes were used, it was found that (*a*) a variable amount of the dye was held up at the beginning of the capillary injection spiral, and (*b*) at very low coronary flow rates, some of the dye passed up the vertical well. To reduce these errors to a minimum, the injection nipple is connected by a short piece of pure gum rubber tubing to a siliconed one-way B-D stopcock. The drugs are injected slowly with the syringe needle inserted through the rubber tubing until the tip is close to the beginning of the injection capillary. Immediately thereafter, 1.5 cc. of perfusate is injected slowly from a second syringe attached to the stopcock through the female Luer connection. After each dose, the injection nipple and capillary are cleared by slowly withdrawing 5 cc. of perfusate.

Cannulation of Hearts.—Removal of the heart of a guinea pig to a dish of perfusate prior to cannulation of the aorta greatly complicated the procedure and led to numerous failures. To overcome this difficulty, the hearts were finally cannulated *in situ* as follows: the glass cannula is connected by a 2-ft. piece of pure gum rubber tubing to the stopcock attached to the injection nipple, and a slow flow of perfusate is started. After exposure of the heart and aorta, the cannula is ligated into the latter in the usual manner. The heart, with cannula in place, is then cut free from its attachments. After detaching the rubber tubing, the cannulated heart is placed in position in the apparatus as quickly as possible.

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[1] Manufactured by Beckman Instruments, Inc., South Pasadena, Calif.

[2] Grateful acknowledgment is made to Mr. W. A. Helbig, Darco Corp., 60 E. 42nd St., New York 17, N. Y., for his suggestions and for placing a supply of the carbon at the disposal of the authors.

REFERENCES

(1) Leitch, J. L., and Haley, T. J., *J. Pharmacol. Exptl. Therap.*, **104**, 416(1952).

(2) Anderson, F. F., and Craver, B. N., *ibid.*, **93**, 135 (1948).

(3) Gilbert, P. T., Jr., *Science*, **114**, 637(1951).

(4) Chenoweth, M. B., and Koelle, E. S., *J. Lab. Clin. Med.*, **31**, 600(1946).

(5) Anderson, F. F., and Cameron, A., THIS JOURNAL, **39**, 183(1950).

Interim Revision Announcement

NATIONAL FORMULARY, NINTH EDITION

Official from November 1, 1952

By virtue of the authority conveyed in Chapter III, Article X and Chapter IX, Article V of the By-Laws of the AMERICAN PHARMACEUTICAL ASSOCIATION, the Council of the Association appointed the Committee on National Formulary, approved the text of this Interim Revision Announcement prepared by the Committee, authorized the printing thereof, and fixed November 1, 1952, as the date upon which it shall become official.

ROBERT P. FISCHELIS, *Secretary*
Council of the A. PH. A.

JUSTIN L. POWERS, *Chairman*
Committee on National Formulary

ADDITIONS, CHANGES, AND CORRECTIONS

The page numbers cited refer to N. F. IX.

Containers, page 6—Add the following sections:

Single-dose Containers—A single-dose container is a hermetic container holding a quantity of drug intended for administration as a single dose, and which when opened cannot be resealed with assurance that sterility has been maintained. The labeling of a single-dose container which is not closed by fusion shall indicate that the contents are intended for administration as a single dose.

Multiple-dose Containers—A multiple-dose container is a hermetic container which permits withdrawal of successive portions of the contents without changing the strength, quality, or purity of the remaining portion.

Ammonium Chloride Tablets, page 42—Change the fourth line of the *Assay* to read:

. . . Chloride, to a 250-cc. glass-stoppered flask, and dissolve in 40 cc. of water, add . . .

Amobarbital Sodium, Sterile, page 50—Change the paragraph entitled *Description and other requirements* to read:

Description and other requirements—Sterile Amobarbital Sodium conforms to the *Description* and meets the *Identification tests* and other requirements under *Amobarbital Sodium*, page 48.

Following the paragraph entitled *Description and other requirements* add:

Completeness of solution—Place 1 Gm. of Sterile Amobarbital Sodium in a 10-cc. glass-stoppered cylinder, nearly fill the cylinder with carbon dioxide-free water, and shake gently until dissolved: the solution is clear.

Calcium Glycerophosphate, page 107, and First. N. F. IX Supplement, page 2—Change the first paragraph of the monograph to read:

Calcium Glycerophosphate is the normal calcium salt of glycerophosphoric acid, and when dried at 150° for 4 hours, it contains not less than 98 per cent of $C_3H_7CaPO_6$.

Change the paragraph entitled *Loss on drying* to read:

Loss on drying—Dry Calcium Glycerophosphate at 150° for 4 hours: it loses not more than 12 per cent of its weight, page 741.

Change the first sentence of the *Assay* to read:

Dry about 400 mg. of Calcium Glycerophosphate at 150° for 4 hours, weigh accurately, dissolve in 100 cc. of water, and add 1 cc. of hydrochloric acid.

Carmine, page 127—Change the paragraph entitled *Residue on ignition* to read:

Residue on ignition—Weigh accurately about 1 Gm. of Carmine and ignite it to constant weight: it yields not more than 12 per cent of residue on ignition, page 759.

Effervescent Powders, Compound, page 183—Change the last sentence in the first paragraph of the monograph to read:

The white paper contains not less than 2 Gm. and not more than 2.4 Gm. of tartaric acid ($C_4H_6O_6$).

Glyceryl Monostearate, page 237—Change the paragraph entitled *Melting range* to read:

Melting range—Glyceryl Monostearate does not melt below 56°.

Grindelia, page 240—Change the paragraph entitled *Stems* to read:

Stems—Grindelia contains not more than 30 per cent of its stems over 2 mm. in diameter.

Honey, page 248—Delete the entire paragraph entitled *Foreign coloring matter,* page 249.

Liver, Desiccated, page 299—Change the first sentence of the monograph to read:

Desiccated Liver is the dried, defatted or undefatted powder prepared from mammalian livers suitable for use as food by man.

After the paragraph entitled *Packaging and storage* add:

Labeling—The label of the container must indicate whether desiccated liver is defatted or undefatted.

Lobelia, page 301—Change the paragraph entitled *Stems* to read:

Stems—Lobelia contains not more than 15 per cent of its stems over 3 mm. in diameter.

Nux Vomica Extract, page 357—Change the first sentence of the *Assay,* beginning in the second line to read:

. . . and 5 cc. of 1 *N* sulfuric acid, or a sufficient amount to render the mixture acid to litmus Evaporate on a . . .

Sodium Borate Solution, Compound, page 473—Change the first sentence of the *Assay* to read:

Transfer 20 cc. of Compound Sodium Borate Solution to an iodine flask and add 25 cc. of water and 50 cc. of 0.1 *N* bromine.

Sodium Hypophosphite, page 483—Change the paragraph entitled *Loss on drying* to read:

Loss on drying—Dry Sodium Hypophosphite over sulfuric acid for 2 hours: It loses not more than 3 per cent of its weight, page 741.

Theobromine, page 539—Change the first sentence of the paragraph entitled *Solubility* to read:

One Gm. of Theobromine is soluble in about 1800 cc. of water, in 2400 cc. of alcohol, and 6000 cc. of chloroform.

Zinc Phenolsulfonate, page 574—Change the last sentence in the *Assay* to read:

Each Gm. of zinc oxide is equivalent to 5.059 Gm. of $C_{12}H_{10}O_8S_2Zn$.

Ferric Citrate, page 667—Replace the statement "Use Ferric Citrate, reagent grade, U.S.P. XIV", by the following monograph:

Ferric Citrate, ($FeC_6H_5O_7.xH_2O$)—Thin, transparent, garnet red scales, or brown granules. Slowly soluble in water, more readily soluble in hot water, the solubility diminishing with age. It is insoluble in alcohol.

Assay—Weigh accurately about 1 Gm. of ferric citrate, dissolve it in a mixture of 5 cc. of reagent hydrochloric acid and 25 cc. of water in a glass-stoppered flask, warming if necessary, to aid solution. Cool, add 4 Gm. of potassium iodide, stopper the flask and allow it to stand for 15 minutes. Dilute it with 100 cc. of water and titrate the liberated iodine with 0.1 *N* sodium thiosulfate, using starch T.S. as the indicator toward the end. Each cc. of 0.1 *N* sodium thiosulfate is equivalent to 5.585 mg. of Fe. It shows not less than 16.5 per cent and not more than 18.5 per cent of Fe.

Chloride—Heat 1 Gm. with 25 cc. of water and 2 cc. of nitric acid until dissolved. Cool, dilute to 100 cc. with water, and mix well. To 10 cc. of the solution add 1 cc. of silver nitrate T.S.: no turbidity is produced at once.

Sulfate—To 10 cc. of the solution obtained in the preceding test, add 1 cc. of barium nitrate T.S.: no turbidity is produced in 15 seconds.

Tartrate—Dissolve 500 mg. by heating it with 15 cc. of water on a water bath, then add to the solution 10 cc. of potassium hydroxide T.S., boil for a few minutes, and filter. To half of the filtrate add 2 cc. of glacial acetic acid, cool, and allow to stand for 1 hour with frequent agitation; no white precipitate, soluble in ammonia T.S., is produced.

Alkali citrate—Ignite about 500 mg. until it is thoroughly charred, cool, and add 2 cc. of hot water The water is neutral or shows only a slight alkaline reaction to litmus paper.

Ammonia—Heat 500 mg. with 5 cc. of sodium hydroxide T.S.: the odor of ammonia is not perceptible.

Sodium Carbonate, Monohydrated, page 683—Change the statement following this title to read: Use *Monohydrated Sodium Carbonate*, N. F. IX.

Sodium Desoxycholate, page 684—Delete the paragraph entitled *Loss on drying*.

Choline Assay, page 698—Change the last sentence of the third paragraph in the *Assay for Choline* to read:

Multiply the choline chloride content by 0.868 to obtain the choline content of the sample being assayed.

Injections, page 732—Add the following to the respective columns, page 738, under the section on *Volumes of Injection in Container:*

30.0 cc. 0.8 cc. 1.2 cc.

Delete the section, page 738, on *Number of Doses in Container*. This deletion is effective immediately.

Add the following section:

Injections

Packaging and Storage—Package the following in single-dose containers. The volume of Injection or Solution in single-dose containers shall not exceed that intended for administration at one time.

Calcium Chloride Injection
Calcium Levulinate Injection
Camphor Injection
Ferric Ammonium Citrate, Green, Injection
Ferric Cacodylate Injection
Histidine Monohydrochloride Injection
Sodium Cacodylate Injection
Sodium Dehydrocholate Injection
Sodium Indigotindisulfonate Injection
Sodium Iodide Injection
Sodium Salicylate and Iodide Injection
Sodium Salicylate and Iodide with Colchicine Injection
Sodium Thiosulfate Injection
Strophanthin Injection

Package the following Injections in either single-dose or multiple-dose containers unless intended for intraspinal, intracisternal, or peridural administration, in which cases single-dose containers shall be used. Unless otherwise specified in the respective monograph, no multiple-dose container shall contain a volume of Injection more than sufficient to permit the withdrawal of 30 cc.

Magnesium Sulfate Injection
Mercuric Salicylate Injection
Mercuric Succinimide Injection
Quinine and Urea Hydrochloride Injection
Quinine Dihydrochloride Injection

Injections intended for veterinary use are exempt from the above-listed requirements.

The above supersedes all present *Packaging and storage* requirements given in the individual monographs for the preparations listed.

Sterility Test for Liquids and Solids, page 768—Change the first paragraph under *Sterility Test Media* to read:

Sterility Test Media—Test Media may be prepared as follows or dehydrated mixtures may be used which, when reconstituted with water as directed by the manufacturer or distributor, have the same composition and yield products conforming to those obtained from the formulas given below. Such products for Thioglycollate Media must have growth-promoting, buffer, and oxygen

tension-controlling properties equal to, or better than, the media prepared from the formulas given below. In preparing Thioglycollate Media avoid contamination with calcium.

Change the third paragraph under *Sterility Test Media* to read:

For 15 cc. of medium use tubes preferably 25 x 150 mm., for 40 cc. of medium use tubes preferably 25 x 200 mm., and for 75 to 100 cc. of medium use tubes preferably 38 x 200 mm.

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Book Reviews

Heterocyclic Compounds. Vol. 3. Edited by ROBERT C. ELDERFIELD. John Wiley & Sons, Inc., New York, 1952. vi + 442 pp. 15.5 x 23.5 cm. Price $12.

Heterocyclic Compounds. Vol. 4. Edited by ROBERT C. ELDERFIELD. John Wiley & Sons, Inc., New York, 1952. vi + 674 pp. 15.5 x 23.5 cm. Price $17.

These two volumes constitute a systematic treatise on the chemistry of polycyclic nitrogen heterocycles containing one hetero nitrogen atom. Volume 3 is concerned with the syntheses, reactions, and derivatives of indole, isoindole, carbazole, pyridine, quinindines, and related compounds. It also includes a chapter on bicyclic systems with a nitrogen atom common to both rings.

About half of Volume 4 is devoted to the chemistry of quinoline, followed by shorter chapters on isoquinoline, the acridines, phenanthridine, and the benzoquinolines.

Originally it was planned to combine these two volumes into one, but it was found that to do this would necessitate an inordinately large book. These volumes constitute a significant contribution to organic chemistry and should be of especial interest to pharmaceutical and medicinal chemists, because of the relationship between several of the compounds discussed and the alkaloids and certain synthetic medicinal chemicals. Detailed discussions of the chemistry of the alkaloids, however, have been omitted, except in the case of those possessing an isoquinoline nucleus where the chemistry of quinoline evolved to such an extent from the study of these alkaloids. References to many excellent treatises on the alkaloids are included.

Both volumes are adequately documented with many references to the original literature. The editor has been very successful in maintaining a uniform style throughout, despite the fact that seven of the ten chapters were contributed by specialists in each main branch coming within the purview of work. The printing and binding are of good quality and reflect the general excellence of the contents of the two volumes.

Terramycin Bibliography 1950–1951. Chas. Pfizer and Co., Inc., Brooklyn, 1952. 81 pp. 15.5 x 23 cm.

This bibliography is designed to furnish experimental and clinical investigators with complete and accurate references to all publications on terramycin. In addition, it lists papers now in press as completely as could be determined at the time of publication of the bibliography.

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BOOKS RECEIVED

Report to the Economic and Social Council on Statistics of Narcotics for 1950 and the Work of the Board in 1951. Issued by United Nations Publications, Geneva, through Columbia University Press, New York, 1951. 28 x 21.5 cm. 68 pp. Price $.60.

Estimated World Requirements of Narcotic Drugs in 1952. Issued by United Nations Publications, Geneva, through Columbia University Press, New York, 1951. 28 x 21.5 cm. 64 pp. Price $.60.

Addendum to the Report to the Economic and Social Council on Statistics of Narcotics for 1950 and the Work of the Board in 1951. Issued by United Nations Publications, Geneva, through Columbia University Press, New York, 1951. 28 x 21.5 cm. 39 pp. Price $.30.

Symposium on Radiobiology. The Basic Aspects of Radiation Effects on Living Systems. Edited by JAMES J. NICKSON. John Wiley & Sons, Inc., New York, 1952. xii + 465 pp. 15.5 x 23.5 cm. Price $7.50.

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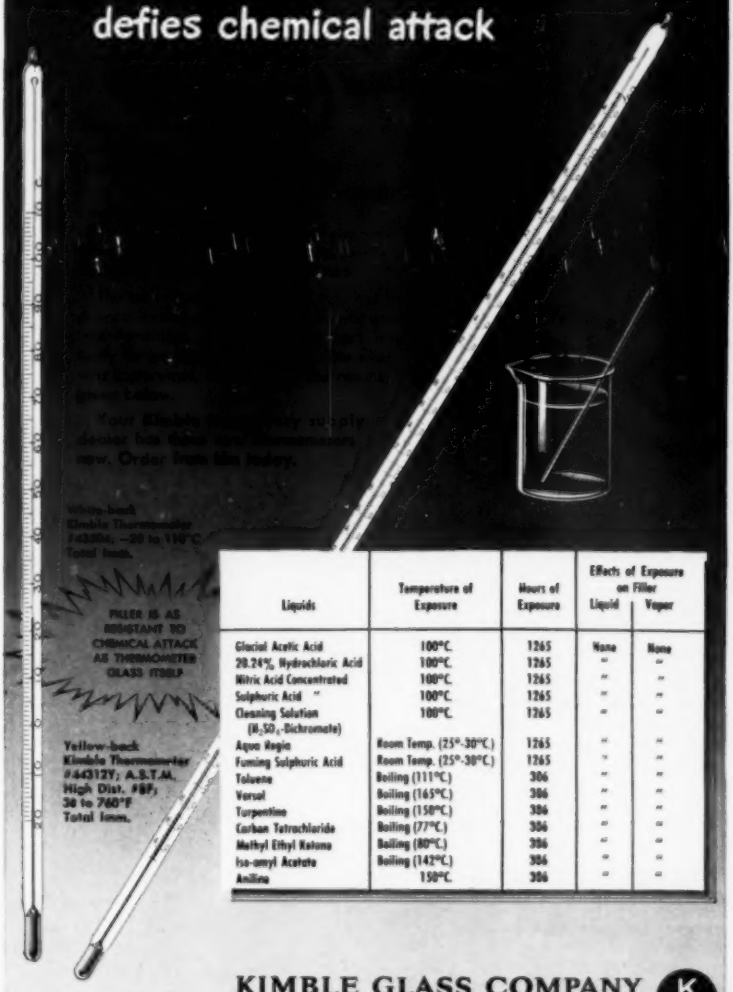
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Nitric Acid Concentrated	100°C.	1265	"	"
Sulphuric Acid "	100°C.	1265	"	"
Cleaning Solution (H_2SO_4-Dichromate)	100°C.	1265	"	"
Aqua Regia	Room Temp. (25°-30°C.)	1265	"	"
Fuming Sulphuric Acid	Room Temp. (25°-30°C.)	1265	"	"
Toluene	Boiling (111°C.)	306	"	"
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